

# Vascular Endothelial Growth Factor 2

## Cross-Reference to Related Applications

*See a1*  
The present application is a continuation-in-part of U.S. Application No. 08/\_\_\_\_ (Attorney Docket No. 1488.1000004), filed December 24, 1997, which is herein incorporated by reference; the present application is also a continuation-in-part of 08/465,968, filed June 6, 1995, which is herein incorporated by reference; said 08/\_\_\_\_ (Attorney Docket No. 1488.1000004) is a continuation-in-part of both of said 08/465,968 and U.S. Application No. 08/207,550, filed March 8, 1994; said 08/465,968 is a continuation-in-part of said 08/207,550, which is also herein incorporated by reference.

## Background of the Invention

### Field of the Invention

The present invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. The polypeptides of the present invention have been identified as members of the vascular endothelial growth factor family. More particularly, the polypeptides of the present invention are human vascular endothelial growth factor 2 (VEGF2). The invention also relates to inhibiting the action of such polypeptides.

### Related Art

The formation of new blood vessels, or angiogenesis, is essential for embryonic development, subsequent growth, and tissue repair. Angiogenesis is also an essential part of certain pathological conditions, such as neoplasia (*i.e.*, tumors and gliomas). Abnormal angiogenesis is associated with other diseases such as inflammation, rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman, J. and Klagsbrun, M., *Science* 235:442-447(1987)).

Both acidic and basic fibroblast growth factor molecules are mitogens for endothelial cells and other cell types. Angiotropin and angiogenin can induce angiogenesis, although their functions are unclear (Folkman, J., *Cancer Medicine*, Lea and Febiger Press, pp. 153-170 (1993)). A highly selective mitogen for vascular endothelial cells is vascular endothelial growth factor or VEGF (Ferrara, N. *et al.*, *Endocr. Rev.* 13:19-32 (1992)), which is also known as vascular permeability factor (VPF).

Vascular endothelial growth factor is a secreted angiogenic mitogen whose target cell specificity appears to be restricted to vascular endothelial cells. The murine VEGF gene has been characterized and its expression pattern in embryogenesis has been analyzed. A persistent expression of VEGF was observed in epithelial cells adjacent to fenestrated endothelium, *e.g.*, in choroid plexus and kidney glomeruli. The data was consistent with a role of VEGF as a multifunctional regulator of endothelial cell growth and differentiation (Breier, G. *et al.*, *Development* 114:521-532 (1992)).

VEGF shares sequence homology with human platelet-derived growth factors, PDGF $\alpha$  and PDGF $\beta$  (Leung, D.W., *et al.*, *Science* 246:1306-1309, (1989)). The extent of homology is about 21% and 23%, respectively. Eight cysteine residues contributing to disulfide-bond formation are strictly conserved in these proteins. Although they are similar, there are specific differences between VEGF and PDGF. While PDGF is a major growth factor for connective tissue, VEGF is highly specific for endothelial cells. Alternatively spliced mRNAs have been identified for both VEGF, PLGF, and PDGF and these different splicing products differ in biological activity and in receptor-binding specificity. VEGF and PDGF function as homo-dimers or hetero-dimers and bind to receptors which elicit intrinsic tyrosine kinase activity following receptor dimerization.

VEGF has four different forms of 121, 165, 189 and 206 amino acids due to alternative splicing. VEGF121 and VEGF165 are soluble and are capable of promoting angiogenesis, whereas VEGF189 and VEGF206 are bound to heparin containing proteoglycans in the cell surface. The temporal and spatial expression

of VEGF has been correlated with physiological proliferation of the blood vessels (Gajdusek, C.M., and Carbon, S.J., *Cell Physiol.* 139:570-579 (1989); McNeil, P.L., *et al.*, *J. Cell. Biol.* 109:811-822 (1989)). Its high affinity binding sites are localized only on endothelial cells in tissue sections (Jakeman, L.B., *et al.*, *Clin. Invest.* 89:244-253 (1989)). The factor can be isolated from pituitary cells and several tumor cell lines, and has been implicated in some human gliomas (Plate, K.H., *Nature* 359:845-848 (1992)). Interestingly, expression of VEGF121 or VEGF165 confers on Chinese hamster ovary cells the ability to form tumors in nude mice (Ferrara, N. *et al.*, *J. Clin. Invest.* 91:160-170 (1993)). The inhibition of VEGF function by anti-VEGF monoclonal antibodies was shown to inhibit tumor growth in immune-deficient mice (Kim, K.J., *Nature* 362:841-844 (1993)). Further, a dominant-negative mutant of the VEGF receptor has been shown to inhibit growth of glioblastomas in mice.

Vascular permeability factor (VPF) has also been found to be responsible for persistent microvascular hyperpermeability to plasma proteins even after the cessation of injury, which is a characteristic feature of normal wound healing. This suggests that VPF is an important factor in wound healing. Brown, L.F. *et al.*, *J. Exp. Med.* 176:1375-1379 (1992).

The expression of VEGF is high in vascularized tissues, (*e.g.*, lung, heart, placenta and solid tumors) and correlates with angiogenesis both temporally and spatially. VEGF has also been shown to induce angiogenesis *in vivo*. Since angiogenesis is essential for the repair of normal tissues, especially vascular tissues, VEGF has been proposed for use in promoting vascular tissue repair (*e.g.*, in atherosclerosis).

U.S. Patent No. 5,073,492, issued December 17, 1991 to Chen *et al.*, discloses a method for synergistically enhancing endothelial cell growth in an appropriate environment which comprises adding to the environment, VEGF, effectors and serum-derived factor. Also, vascular endothelial cell growth factor C sub-unit DNA has been prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a heterodimer or homodimer. The

protein is a mammalian vascular endothelial cell mitogen and, as such, is useful for the promotion of vascular development and repair, as disclosed in European Patent Application No. 92302750.2, published September 30, 1992.

### *Summary of the Invention*

5 The polypeptides of the present invention have been putatively identified as a novel vascular endothelial growth factor based on amino acid sequence homology to human VEGF.

In accordance with one aspect of the present invention, there are provided novel mature polypeptides, as well as biologically active and diagnostically or therapeutically useful fragments, analogs, and derivatives thereof. The polypeptides of the present invention are of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules comprising polynucleotides encoding full length or truncated VEGF2 polypeptides having the amino acid sequences shown in SEQ ID NOS:2 or 4, respectively, or the amino acid sequences encoded by the cDNA clones deposited in bacterial hosts as ATCC Deposit Number 97149 on May 12, 1995 or ATCC Deposit Number 75698 on March 4, 1994

The present invention also relates to biologically active and diagnostically or therapeutically useful fragments, analogs, and derivatives of VEGF2.

20 In accordance with still another aspect of the present invention, there are provided processes for producing such polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of the present invention, under conditions promoting expression of said proteins and subsequent recovery of said proteins.

25 In accordance with yet a further aspect of the present invention, there are provided processes for utilizing such polypeptides, or polynucleotides encoding such polypeptides for therapeutic purposes, for example, to stimulate

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angiogenesis, wound-healing, growth of damaged bone and tissue, and to promote vascular tissue repair.

In accordance with yet another aspect of the present invention, there are provided antibodies against such polypeptides and processes for producing such polypeptides.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, to prevent tumor angiogenesis and thus inhibit the growth of tumors, to treat diabetic retinopathy, inflammation, rheumatoid arthritis and psoriasis.

In accordance with another aspect of the present invention, there are provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to nucleic acid sequences of the present invention.

In accordance with another aspect of the present invention, there are provided methods of diagnosing diseases or a susceptibility to diseases related to mutations in nucleic acid sequences of the present invention and proteins encoded by such nucleic acid sequences.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

### ***Brief Description of the Figures***

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

FIGS. 1A-1E show the full length nucleotide (SEQ ID NO:1) and the deduced amino acid (SEQ ID NO:2) sequence of VEGF2. The polypeptide comprises approximately 419 amino acid residues of which approximately 23 represent the leader sequence. The standard one letter abbreviations for amino acids are used. Sequencing was performed using the Model 373 Automated DNA Sequencer (Applied Biosystems, Inc.). Sequencing accuracy is predicted to be greater than 97%.

FIGS. 2A-2D show the nucleotide (SEQ ID NO:3) and the deduced amino acid (SEQ ID NO:4) sequence of a truncated, biologically active form of VEGF2. The polypeptide comprises approximately 350 amino acid residues of which approximately the first 24 amino acids represent the leader sequence.

FIGS. 3A-3B are an illustration of the amino acid sequence homology between PDGF $\alpha$  (SEQ ID NO:5), PDGF $\beta$  (SEQ ID NO:6), VEGF (SEQ ID NO:7), and VEGF2 (SEQ ID NO:4). The boxed areas indicate the conserved sequences and the location of the eight conserved cysteine residues.

FIG. 4 shows, in table-form, the percent homology between PDGF $\alpha$ , PDGF $\beta$ , VEGF, and VEGF2.

FIG. 5 shows the presence of VEGF2 mRNA in human breast tumor cell lines.

FIG. 6 depicts the results of a Northern blot analysis of VEGF2 in human adult tissues.

FIG. 7 shows a photograph of an SDS-PAGE gel after *in vitro* transcription, translation and electrophoresis of the polypeptide of the present invention. Lane 1:  $^{14}$ C and rainbow M.W. marker; Lane 2: FGF control; Lane 3: VEGF2 produced by M13-reverse and forward primers; Lane 4: VEGF2 produced by M13 reverse and VEGF-F4 primers; Lane 5: VEGF2 produced by M13 reverse and VEGF-F5 primers.

FIGS. 8A and 8B depict photographs of SDS-PAGE gels. VEGF2 polypeptide was expressed in a baculovirus system consisting of Sf9 cells. Protein

from the medium and cytoplasm of cells were analyzed by SDS-PAGE under non-reducing (FIG.8A) and reducing (FIG.8B) conditions.

FIG. 9 depicts a photograph of an SDS-PAGE gel. The medium from Sf9 cells infected with a nucleic acid sequence of the present invention was precipitated. The resuspended precipitate was analyzed by SDS-PAGE and stained with coomassie brilliant blue.

FIG. 10 depicts a photograph of an SDS-PAGE gel. VEGF2 was purified from the medium supernatant and analyzed by SDS-PAGE in the presence or absence of the reducing agent  $\beta$ -mercaptoethanol and stained by coomassie brilliant blue.

FIG. 11 depicts reverse phase HPLC analysis of purified VEGF2 using a RP-300 column (0.21 x 3 cm, Applied Biosystems, Inc.). The column was equilibrated with 0.1% trifluoroacetic acid (Solvent A) and the proteins eluted with a 7.5 min gradient from 0 to 60% Solvent B, composed of acetonitrile containing 0.07% TFA. The protein elution was monitored by absorbance at 215 nm ("red" line) and 280 nm ("blue" line). The percentage of Solvent B is shown by the "green" line.

FIG. 12 is a bar graph illustrating the effect of partially-purified VEGF2 protein on the growth of vascular endothelial cells in comparison to basic fibroblast growth factor.

FIG. 13 is a bar graph illustrating the effect of purified VEGF2 protein on the growth of vascular endothelial cells.

FIGS. 14A and 14B  
FIG. 14 depicts expression of VEGF2 mRNA in human fetal and adult tissues.

FIG. 15 depicts expression of VEGF2 mRNA in human primary culture cells.

FIGS. 16A and 16B

FIG. 16 depicts transient expression of VEGF2 protein in COS-7 cells.

FIG. 17 depicts VEGF2 stimulated proliferation of human umbilical vein endothelial cells (HUVEC).

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FIG. 18 depicts VEGF2 stimulated proliferation of dermal microvascular endothelial cells.

FIG. 19 depicts the stimulatory effect of VEGF2 on proliferation of microvascular, umbilical cord, endometrial, and bovine aortic endothelial cells.

FIGS. 20A and 20B

~~FIG. 20~~ depicts inhibition of PDGF-induced vascular (human aortic) smooth muscle cell proliferation.

FIGS. 21A and 21B

~~FIG. 21~~ depicts stimulation of migration of HUVEC and bovine microvascular endothelial cells (BMEC) by VEGF-2.

FIG. 22 depicts stimulation of nitric oxide release of HUVEC by VEGF-2 and VEGF-1.

FIG. 23 depicts inhibition of cord formation of microvascular endothelial cells (CADMEC) by VEGF-2.

FIG. 24 depicts stimulation of angiogenesis by VEGF, VEGF-2, and bFGF in the CAM assay.

FIGS. 25A - 25D

~~FIG. 25~~ depicts restoration of certain parameters in the ischemic limb by VEGF2 protein (FIG. 25, top panels) and naked expression plasmid (FIG. 25, middle panels): BP ratio (FIG. 25a); Blood Flow and Flow Reserve (FIG. 25b); Angiographic Score (FIG. 25c); Capillary density (FIG. 25d).

FIGS. 26 A-G depicts ability of VEGF2 to affect the diastolic blood pressure in spontaneously hypertensive rats (SHR). FIGS. 26a and b depict the dose-dependent decrease in diastolic blood pressure achieved with VEGF2. (FIGS. 26c and d depict the decreased mean arterial pressure (MAP) observed with VEGF2. Panel E shows the effect of increasing doses of VEGF-2 on the mean arterial pressure (MAP) of SHR rats. Panel F shows the effect of VEGF-2 on the diastolic pressure of SHR rats. Panel G shows the effect of VEGF-2 on the diastolic blood pressure of SHR rats.

FIG. 27 depicts inhibition of VEGF-2N' and VEGF-2-induced proliferation.

FIG. 28 shows a schematic representation of the pHE4a expression vector (SEQ ID NO:16). The locations of the kanamycin resistance marker gene, the

multiple cloning site linker region, the oriC sequence, and the *lacIq* coding sequence are indicated.

FIG. 29 shows the nucleotide sequence of the regulatory elements of the pHE4a promoter (SEQ ID NO:17). The two *lac* operator sequences, the Shine-Delgarno sequence (S/D), and the terminal *Hind*III and *Nde*I restriction sites (italicized) are indicated.

### ***Detailed Description of the Preferred Embodiments***

In accordance with one aspect of the present invention, there are provided isolated nucleic acid molecules comprising a polynucleotide encoding a VEGF2 polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2), which was determined by sequencing a cloned cDNA. The nucleotide sequence shown in SEQ ID NO:1 was obtained by sequencing a cDNA clone, which was deposited on May 12, 1995 at the American Type Tissue Collection (ATCC), 12301 Park Lawn Drive, Rockville, Maryland 20852, and given ATCC Deposit No. 97149.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules comprising a polynucleotide encoding a truncated VEGF2 polypeptide having the deduced amino acid sequence of Figure 2 (SEQ ID NO:4), which was determined by sequencing a cloned cDNA. The nucleotide sequence shown in SEQ ID NO:3 was obtained by sequencing a cDNA clone, which was deposited on March 4, 1994 at the American Type Tissue Collection (ATCC), 12301 Park Lawn Drive, Rockville, Maryland 20852, and given ATCC Deposit Number 75698.

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above.

Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

A polynucleotide encoding a polypeptide of the present invention may be obtained from early stage human embryo (week 8 to 9) osteoclastomas, adult heart or several breast cancer cell lines. The polynucleotide of this invention was discovered in a cDNA library derived from early stage human embryo week 9. It is structurally related to the VEGF/PDGF family. It contains an open reading frame encoding a protein of about 419 amino acid residues of which approximately the first 23 amino acid residues are the putative leader sequence such that the mature protein comprises 396 amino acids, and which protein exhibits the highest amino acid sequence homology to human vascular endothelial growth factor (30% identity), followed by PDGF $\alpha$  (24%) and PDGF $\beta$  (22%). (See FIG. 4). It is particularly important that all eight cysteines are conserved within all four members of the family (see boxed areas of FIG. 3). In addition, the signature for the PDGF/VEGF family, PXCXXXXRCXGCCN, (SEQ ID NO: 8) is conserved in VEGF2 (see FIG. 3). The homology between VEGF2, VEGF and the two PDGFs is at the protein sequence level. No nucleotide sequence homology can be detected, and therefore, it would be difficult to isolate the VEGF2 through simple approaches such as low stringency hybridization.

5 The VEGF2 polypeptide of the present invention is meant to include the full length polypeptide and polynucleotide sequence which encodes for any leader sequences and for active fragments of the full length polypeptide. Active fragments are meant to include any portions of the full length amino acid sequence which have less than the full 419 amino acids of the full length amino acid sequence as shown in SEQ ID NO:2, but still contain the eight cysteine residues shown conserved in Figure 3 and that still have VEGF2 activity.

There are at least two alternatively spliced VEGF2 mRNA sequences present in normal tissues. The two bands in FIG. 7, lane 5 indicate the presence of the alternatively spliced mRNA encoding the VEGF2 polypeptide of the present invention.

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The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in FIG. 1 or FIG. 2, or that of the deposited clones, or may be a different coding sequence which, as a result of the redundancy or degeneracy of the genetic code, encodes the same, mature polypeptide as the DNA of FIG. 1, FIG. 2, or the deposited cDNAs.

20 The polynucleotide which encodes for the mature polypeptide of FIG. 1 or FIG. 2 or for the mature polypeptides encoded by the deposited cDNAs may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequences such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequences) and non-coding sequences, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

25 Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequences for the polypeptide as well

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as a polynucleotide which includes additional coding and/or non-coding sequences.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs, and derivatives of the polypeptide having the deduced amino acid sequence of FIGS. 1 or 2, or the polypeptide encoded by the cDNA of the deposited clones. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in FIGS. 1 or 2 or the same mature polypeptide encoded by the cDNA of the deposited clones as well as variants of such polynucleotides which variants encode for a fragment, derivative, or analog of the polypeptides of FIGS. 1 or 2, or the polypeptide encoded by the cDNA of the deposited clones. Such nucleotide variants include deletion variants, substitution variants, and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in FIGS. 1 or 2, or of the coding sequence of the deposited clones. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an



inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, *e.g.* COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., *et al.*, *Cell* 37:767 (1984)).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 396 in SEQ ID NO:2; (d) a nucleotide sequence encoding the polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No.97149; (e) a nucleotide sequence encoding the mature VEGF2 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No.97149; or (f) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), or (e).

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a

5 nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:4; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:4, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions from about 1 to about 326 in SEQ ID NO:4; (d) a nucleotide sequence encoding the polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No.75698; (e) a nucleotide sequence encoding the mature VEGF2 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No.75698; or (f) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), or (e).

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25 By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a VEGF2 polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the VEGF2 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

30 As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NOS:1 or 3, or to the nucleotides sequence of the deposited cDNA clone(s) can be determined conventionally using known

computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

As described in detail below, the polypeptides of the present invention can be used to raise polyclonal and monoclonal antibodies, which are useful in diagnostic assays for detecting VEGF-2 protein expression as described below or as agonists and antagonists capable of enhancing or inhibiting VEGF-2 protein function. Further, such polypeptides can be used in the yeast two-hybrid system to "capture" VEGF-2 protein binding proteins which are also candidate agonist and antagonist according to the present invention. The yeast two hybrid system is described in Fields and Song, *Nature* 340:245-246 (1989).

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide of the invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. These immunogenic epitopes are believed to be confined to a few loci on the molecule. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen et al., *Proc. Natl. Acad. Sci. USA* 81:3998-4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R. A. (1983) Antibodies that react with predetermined sites on proteins. *Science* 219:660-666. Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals. Peptides that are extremely hydrophobic and those of six or fewer residues generally are ineffective at inducing antibodies that bind to the mimicked protein; longer, soluble peptides, especially those containing proline residues, usually are effective. Sutcliffe et al., *supra*, at 661. For instance, 18 of 20 peptides designed according to these guidelines, containing 8-39 residues covering 75% of the sequence of the influenza virus hemagglutinin HA1 polypeptide chain, induced antibodies that reacted with the HA1 protein or intact virus; and 12/12 peptides from the MuLV polymerase and 18/18 from the rabies glycoprotein induced antibodies that precipitated the respective proteins.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Thus, a high proportion of hybridomas obtained by fusion of spleen cells from donors immunized with an antigen epitope-bearing peptide generally secrete antibody reactive with the native protein. Sutcliffe et al., *supra*, at 663. The antibodies raised by antigenic epitope-bearing peptides or polypeptides are useful to detect the mimicked protein, and antibodies to different peptides may be used for tracking the fate of various regions of a protein precursor which undergoes post-translational processing. The peptides and anti-peptide antibodies may be used in a variety of qualitative or

quantitative assays for the mimicked protein, for instance in competition assays since it has been shown that even short peptides (e.g., about 9 amino acids) can bind and displace the larger peptides in immunoprecipitation assays. See, for instance, Wilson et al., Cell 37:767-778 (1984) at 777. The anti-peptide antibodies of the invention also are useful for purification of the mimicked protein, for instance, by adsorption chromatography using methods well known in the art.

Antigenic epitope-bearing peptides and polypeptides of the invention designed according to the above guidelines preferably contain a sequence of at least seven, more preferably at least nine and most preferably between about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of a polypeptide of the invention, containing about 30, 40, 50, 60, 70, 80, 90, 100, or 150 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are considered epitope-bearing peptides or polypeptides of the invention and also are useful for inducing antibodies that react with the mimicked protein. Preferably, the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (i.e., the sequence includes relatively hydrophilic residues and highly hydrophobic sequences are preferably avoided); and sequences containing proline residues are particularly preferred.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate VEGF-2-specific antibodies include the following: a polypeptide comprising amino acid residues from about leu-37 to about glu-45 in SEQ ID NO:2, from about Tyr-58 to about Gly-66 in SEQ ID NO:2, from about Gln-73 to about Glu-81 in SEQ ID NO:2, from about Asp-100 to about Cys-108 in SEQ ID NO:2, from about Gly-140 to about Leu-148 in SEQ ID NO:2, from about Pro-168 to about Val-176 in SEQ ID NO:2, from about His-183 to about Lys-191 in SEQ ID NO:2, from about Ile-201 to about Thr-209 in SEQ ID NO:2, from about Ala-216 to about Tyr-224 in SEQ ID NO:2, from about Asp-244 to about

His-254 in SEQ ID NO:2, from about Gly-258 to about Glu-266 in SEQ ID NO:2, from about Cys-272 to about Ser-280 in SEQ ID NO:2, from about Pro-283 to about Ser-291 in SEQ ID NO:2, from about Cys-296 to about Gln-304 in SEQ ID NO:2, from about Ala-307 to about Cys-316 in SEQ ID NO:2, from about Val-319 to about Cys-335 in SEQ ID NO:2, from about Cys-339 to about Leu-347 in SEQ ID NO:2, from about Cys-360 to about Glu-373 in SEQ ID NO:2, from about Tyr-378 to about Val-386 in SEQ ID NO:2, and from about Ser-388 to about Ser-396 in SEQ ID NO:2. These polypeptide fragments have been determined to bear antigenic epitopes of the VEGF-2 protein by the analysis of the Jameson-Wolf antigenic index.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means for making peptides or polypeptides including recombinant means using nucleic acid molecules of the invention. For instance, a short epitope-bearing amino acid sequence may be fused to a larger polypeptide which acts as a carrier during recombinant production and purification, as well as during immunization to produce anti-peptide antibodies. Epitope-bearing peptides also may be synthesized using known methods of chemical synthesis. For instance, Houghten has described a simple method for synthesis of large numbers of peptides, such as 10-20 mg of 248 different 13 residue peptides representing single amino acid variants of a segment of the HA1 polypeptide which were prepared and characterized (by ELISA-type binding studies) in less than four weeks. Houghten, R. A. (1985) General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. Proc. Natl. Acad. Sci. USA 82:5131-5135. This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten et al. (1986). In this procedure the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods.

A completely manual procedure allows 500-1000 or more syntheses to be conducted simultaneously. Houghten et al., *supra*, at 5134.

Epitope-bearing peptides and polypeptides of the invention are used to induce antibodies according to methods well known in the art. See, for instance, Sutcliffe et al., *supra*; Wilson et al., *supra*; Chow, M. et al., *Proc. Natl. Acad. Sci. USA* 82:910-914; and Bittle, F. J. et al., *J. Gen. Virol.* 66:2347-2354 (1985). Generally, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling of the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine may be coupled to carrier using a linker such as m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carrier using a more general linking agent such as glutaraldehyde. Animals such as rabbits, rats and mice are immunized with either free or carrier-coupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100 µg peptide or carrier protein and Freund's adjuvant. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

Immunogenic epitope-bearing peptides of the invention, i.e., those parts of a protein that elicit an antibody response when the whole protein is the immunogen, are identified according to methods known in the art. For instance, Geysen et al., *supra*, discloses a procedure for rapid concurrent synthesis on solid supports of hundreds of peptides of sufficient purity to react in an enzyme-linked immunosorbent assay. Interaction of synthesized peptides with antibodies is then easily detected without removing them from the support. In this manner a peptide bearing an immunogenic epitope of a desired protein may be identified routinely

by one of ordinary skill in the art. For instance, the immunologically important epitope in the coat protein of foot-and-mouth disease virus was located by Geysen et al. with a resolution of seven amino acids by synthesis of an overlapping set of all 208 possible hexapeptides covering the entire 213 amino acid sequence of the protein. Then, a complete replacement set of peptides in which all 20 amino acids were substituted in turn at every position within the epitope were synthesized, and the particular amino acids conferring specificity for the reaction with antibody were determined. Thus, peptide analogs of the epitope-bearing peptides of the invention can be made routinely by this method. U.S. Patent No. 4,708,781 to Geysen (1987) further describes this method of identifying a peptide bearing an immunogenic epitope of a desired protein.

Further still, U.S. Patent No. 5,194,392 to Geysen (1990) describes a general method of detecting or determining the sequence of monomers (amino acids or other compounds) which is a topological equivalent of the epitope (i.e., a "mimotope") which is complementary to a particular paratope (antigen binding site) of an antibody of interest. More generally, U.S. Patent No. 4,433,092 to Geysen (1989) describes a method of detecting or determining a sequence of monomers which is a topographical equivalent of a ligand which is complementary to the ligand binding site of a particular receptor of interest. Similarly, U.S. Patent No. 5,480,971 to Houghten, R. A. et al. (1996) on Peralkylated Oligopeptide Mixtures discloses linear C<sub>1</sub>-C<sub>7</sub>-alkyl peralkylated oligopeptides and sets and libraries of such peptides, as well as methods for using such oligopeptide sets and libraries for determining the sequence of a peralkylated oligopeptide that preferentially binds to an acceptor molecule of interest. Thus, non-peptide analogs of the epitope-bearing peptides of the invention also can be made routinely by these methods.

As one of skill in the art will appreciate, VEGF-2 polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and



show an increased half-life in vivo. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker et al., Nature 331:84- 86 (1988)).

5 In accordance with the present invention, novel variants of VEGF-2 are also described. These can be produced by deleting or substituting one or more amino acids of VEGF-2. Natural mutations are called allelic variations. Allelic variations can be silent (no change in the encoded polypeptide) or may have altered amino acid sequence.

10 In order to attempt to improve or alter the characteristics of native VEGF-2, protein engineering may be employed. Recombinant DNA technology known to those skilled in the art can be used to create novel polypeptides. Muteins and deletions can show, e.g., enhanced activity or increased stability. In addition, they could be purified in higher yield and show better solubility at least under certain purification and storage conditions. Set forth below are examples of mutations that can be constructed.

#### *Amino terminal and carboxy terminal deletions*

20 Furthermore, VEGF-2 appears to be proteolytically cleaved upon expression resulting in polypeptide fragments of the following sizes when run on a SDS-PAGE gel (sizes are approximate) (See, Figures 6-8, for example): 80, 59, 45, 43, 41, 40, 39, 38, 37, 36, 31, 29, 21, and 15 kDa. These polypeptide fragments are the result of proteolytic cleavage at both the N-terminal and C-terminal portions of the protein. These proteolytically generated fragments appears to have activity, particularly the 21 kDa fragment.

25 In addition, protein engineering may be employed in order to improve or alter one or more characteristics of native VEGF-2. The deletion of carboxyterminal amino acids can enhance the activity of proteins. One example is interferon gamma that shows up to ten times higher activity by deleting ten amino

acid residues from the carboxy terminus of the protein (Döbeli et al., J. of Biotechnology 7:199-216 (1988)). Thus, one aspect of the invention is to provide polypeptide analogs of VEGF-2 and nucleotide sequences encoding such analogs that exhibit enhanced stability (e.g., when exposed to typical pH, thermal conditions or other storage conditions) relative to the native VEGF-2 polypeptide.

Particularly preferred VEGF-2 polypeptides are shown below (numbering starts with the first amino acid in the protein (Met) (Figure 1 (SEQ ID NO:18)):

Ala (residue 24) -- Ser (residue 419)	Glu (32) -- Ser (419)
Pro (25) -- Ser (419)	Ser (33) -- Ser (419)
Ala (26) -- Ser (419)	Gly (34) -- Ser (419)
Ala (27) -- Ser (419)	Leu (35) -- Ser (419)
Ala (28) -- Ser (419)	Asp (36) -- Ser (419)
Ala (29) -- Ser (419)	Leu (37) -- Ser (419)
Ala (30) -- Ser (419)	Ser (38) -- Ser (419)
Phe (31) -- Ser (419)	Asp (39) -- Ser (419)
	Ala (40) -- Ser (419)
Glu (41) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Gln (414)
Pro (42) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Trp (413)
Asp (43) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Tyr (412)
Ala (44) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Ser (411)
Gly (45) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Pro (410)
Glu (46) -- Ser (419)	Met (1), Glu (23), or Ala(24) -- Val (409)
Ala (47) -- Ser (419)	Met (1), Glu (23), or Ala (24) -- Cys (408)
Thr (48) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Arg (407)
Ala (49) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Cys (406)
Tyr (50) -- Ser (419)	Met (1), Glu (23), or Ala (24) -- Val (405)
Ser (52) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Glu (404)
Asp (54) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Glu (403)
Val (62) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Ser (402)
Val (65) -- Ser (419)	Met(1), Glu (23), or Ala (24) -- Gly (398)
Met(1), Glu (23), or Ala (24) -- Met (418)	Met(1), Glu (23), or Ala (24) -- Pro (397)
Met (1), Glu (23), or Ala (24) -- Gln (417)	Met(1), Glu (23), or Ala (24) -- Lys (393)
Met (1), Glu (23), or Ala (24) -- Pro (416)	Met(1), Glu (23), or Ala (24) -- Met(263)
Met(1), Glu (23), or Ala (24) -- Arg (415)	

Met(1), Glu (23), or Ala (24) - Asp(311)

Met(1), Glu (23), or Ala (24) - Pro (367)

Met(1) - Ser(419)

Met(1) - Ser(228)

Glu(47) - Ser(419)

Ala(111) - Lys(214)

Ala(112) - Lys(214)

His(113) - Lys(214)

Tyr(114) - Lys(214)

Asn(115) - Lys(214)

Thr(116) - Lys(214)

Thr(103) - Leu(215)

Glu(104) - Leu(215)

Glu(105) - Leu(215)

Thr(106) - Leu(215)

Ile(107) - Leu(215)

Lys(108) - Leu(215)

Phe(109) - Leu(215)

Ala(110) - Leu(215)

Ala(111) - Leu(215)

Ala(112) - Leu(215)

His(113) - Leu(215)

Tyr(114) - Leu(215)

Asn(115) - Leu(215)

Thr(116) - Leu(215)

Thr(103) - Ser(228)

Glu(104) - Ser(228)

Glu(105) - Ser(228)

Thr(106) - Ser(228)

Ile(107) - Ser(228)

Lys(108) - Ser(228)

Phe(109) - Ser(228)

Ala(110) - Ser(228)

Ala(111) - Ser(228)

Ala(112) - Ser(228)

His(113) - Ser(228)

Tyr(114) - Ser(228)

Asn(115) - Ser(228)

Thr(116) - Ser(228)

Thr(103) - Leu(229)

Glu(104) - Leu(229)

Thr(103) - Arg(227)

Glu(104) - Arg(227)

Glu(105) - Arg (227)

Thr(106) - Arg (227)

Ile(107) - Arg (227)

Lys(108) - Arg (227)

Phe(109) - Arg (227)

Ala(110) - Arg (227)

Ala(111) - Arg (227)

Ala(112) - Arg (227)

His(113) - Arg (227)

Tyr(114) - Arg (227)

Asn(115) - Arg (227)

Thr(116) - Arg (227)

Thr(103) - Ser(213)

Glu(104) - Ser(213)

Glu(105) - Ser(213)

Thr(106) - Ser(213)

Ile(107) - Ser(213)

Lys(108) - Ser(213)

Phe(109) - Ser(213)

Ala(110) - Ser(213)

Ala(111) - Ser(213)

Ala(112) - Ser(213)

His(113) - Ser(213)

Tyr(114) - Ser(213)

Asn(115) - Ser(213)

Thr(116) - Ser(213)

Thr(103) - Lys(214)

Glu(104) - Lys(214)

Glu(105) - Lys(214)

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Thr(106) -- Lys(214)  
 Ile(107) -- Lys(214)  
 Lys(108) -- Lys(214)  
 Phe(109) -- Lys(214)  
 5 Ala(110) -- Lys(214)  
 Glu(105) -- Leu(229)  
 Thr(106) -- Leu(229)  
 Ile(107) -- Leu(229)  
 Lys(108) -- Leu(229)  
 10 Phe(109) -- Leu(229)  
 Ala(110) -- Leu(229)  
 Ala(111) -- Leu(229)  
 Ala(112) -- Leu(229)  
 His(113) -- Leu(229)  
 Tyr(114) -- Leu(229)  
 Asn(115) -- Leu(229)  
 Thr(116) -- Leu(229)

Preferred embodiments include the following deletion mutants: Thr(103)  
 -- Arg(227); Glu(104) -- Arg(227); Ala(112) -- Arg (227); Thr(103) -- Ser(213);  
 Glu(104) -- Ser(213); Thr(103) -- Leu(215); Glu(47) -- Ser(419); Met(1), Glu  
 (23), or Ala (24) -- Met(263); Met(1), Glu (23), or Ala (24) -- Asp(311); Met(1),  
 Glu (23), or Ala (24) -- Pro (367); Met(1) -- Ser(419); and Met(1) -- Ser(228) of  
 (Figure 1 (SEQ ID NO:18)).

Also included by the present invention are deletion mutants having amino  
 25 acids deleted from both the N-terminus and the C-terminus. Such mutants include  
 all combinations of the N-terminal deletion mutants and C-terminal deletion  
 mutants described above. Those combinations can be made using recombinant  
 techniques known to those skilled in the art.

Thus, in one aspect, N-terminal deletion mutants are provided by the  
 30 present invention. Such mutants include those comprising the amino acid  
 sequence shown in Figure 1 (SEQ ID NO:18) except for a deletion of at least the

first 24 N-terminal amino acid residues (i.e., a deletion of at least Met (1) -- Glu (24)) but not more than the first 115 N-terminal amino acid residues of Figure 1 (SEQ ID NO:18). Alternatively, first 24 N-terminal amino acid residues (i.e., a deletion of at least Met (1) -- Glu (24)) but not more than the first 103 N-terminal amino acid residues of Figure 1 (SEQ ID NO:18), etc, etc.

In another aspect, C-terminal deletion mutants are provided by the present invention. Such mutants include those comprising the amino acid sequence shown in Figure 1 (SEQ ID NO:18) except for a deletion of at least the last C-terminal amino acid residue (Ser (419)) but not more than the last 220 C-terminal amino acid residues (i.e., a deletion of amino acid residues Val (199) - Ser (419)) of Figure 1 (SEQ ID NO:18). Alternatively, the deletion will include at least the last C-terminal amino acid residue but not more than the last 216 C-terminal amino acid residues of Figure 1 (SEQ ID NO:18). Alternatively, the deletion will include at least the last C-terminal amino acid residue but not more than the last 204 C-terminal amino acid residues of Figure 1 (SEQ ID NO:18). Alternatively, the deletion will include at least the last C-terminal amino acid residues but not more than the last 192 C-terminal amino acid residues of Figure 1 (SEQ ID NO:18). Alternatively, the deletion will include at least the last C-terminal amino acid residues but not more than the last 156 C-terminal amino acid residues of Figure 1 (SEQ ID NO:18). Alternatively, the deletion will include at least the last C-terminal amino acid residues but not more than the last 108 C-terminal amino acid residues of Figure 1 (SEQ ID NO:18). Alternatively, the deletion will include at least the last C-terminal amino acid residues but not more than the last 52 C-terminal amino acid residues of Figure 1 (SEQ ID NO:18).

In yet another aspect, also included by the present invention are deletion mutants having amino acids deleted from both the N-terminal and C-terminal residues. Such mutants include all combinations of the N-terminal deletion mutants and C-terminal deletion mutants described above.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region

(leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having the nucleotide sequence of the deposited cDNA(s) or the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:3 is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt in length which are useful as diagnostic probes and primers as discussed herein. Of course, larger fragments of 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1025, 1050, 1075, 1100, 1125, 1150, 1175, 1200, 1225, 1250, 1275, 1300, 1325, 1350, 1375, 1400, 1425, 1450, 1475, 1500, 1525, 1550, 1575, 1600, 1625, 1650 or 1674 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of the deposited cDNA(s) or as shown in SEQ ID NO:1 or SEQ ID NO:3. By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of the deposited cDNA(s) or the nucleotide sequence as shown in SEQ ID NOS:1 or 3.

Moreover, representative examples of VEGF2 polynucleotide fragments include, for example, fragments having a sequence from about nucleotide number 1-50, 51-100, 101-150, 151-200, 201-250, 251-300, 301-350, 351-400, 401-450, 451-500, 501-550, 551-600, 651-700, 701-750, 751-800, 800-850, 851-900, 901-950, or 951 to the end of SEQ ID NO:1 or the cDNA contained in the deposited clone. In this context "about" includes the particularly recited ranges, larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini. Preferably, these fragments encode a polypeptide which has biological activity.

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Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

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A VEGF2 "polynucleotide" also includes those polynucleotides capable of hybridizing, under stringent hybridization conditions, to sequences contained in SEQ ID NO:1 or for instance, the cDNA clone(s) contained in ATCC Deposit Nos. 97149 or 75698, the complement thereof. "Stringent hybridization conditions" refers to an overnight incubation at 42° C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

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Also contemplated are nucleic acid molecules that hybridize to the VEGF2 polynucleotides at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH<sub>2</sub>PO<sub>4</sub>; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking

DNA; followed by washes at 50°C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC).

Note that variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

Of course, a polynucleotide which hybridizes only to polyA<sup>+</sup> sequences (such as any 3' terminal polyA<sup>+</sup> tract of a cDNA shown in the sequence listing), or to a complementary stretch of T (or U) residues, would not be included in the definition of "polynucleotide," since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the deposited cDNA or the nucleotide sequence as shown in SEQ ID NO:1). Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of the VEGF2 cDNA shown in SEQ ID NOS:1 or 3), or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would



hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

The present application is directed to nucleic acid molecules at least 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NOS:1 or 3 or to the nucleic acid sequence of the deposited cDNA(s), irrespective of whether they encode a polypeptide having VEGF2 activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having VEGF2 activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having VEGF2 activity include, *inter alia*, (1) isolating the VEGF2 gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the VEGF2 gene, as described in Verma *et al.*, *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and Northern Blot analysis for detecting VEGF2 mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence shown in SEQ ID NOS:1 or 3 or to a nucleic acid sequence of the deposited cDNA(s) which do, in fact, encode a polypeptide having VEGF2 protein activity. By "a polypeptide having VEGF2 activity" is intended polypeptides exhibiting VEGF2 activity in a particular biological assay. For example, VEGF2 protein activity can be measured using, for example, mitogenic assays and endothelial cell migration assays. See, e.g., Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA* 93:2576-2581 (1996) and Joukov *et al.*, *EMBO J.* 5:290-298 (1996).

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to a nucleic acid sequence of the deposited cDNA(s) or the nucleic acid

sequence shown in SEQ ID NO:1 or SEQ ID NO:3 will encode a polypeptide "having VEGF2 protein activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having VEGF2 protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95%, 96%, 97%, or 98% identity to a polynucleotide which encodes the polypeptides of SEQ ID NOS:2 or 4, as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

"Identity" per se has an art-recognized meaning and can be calculated using published techniques. (See, e.g.: (COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A.M., ed., Oxford University Press, New York, (1988); BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed., Academic Press, New York, (1993); COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, (1994); SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heinje, G., Academic Press, (1987); and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds., M Stockton Press,

New York, (1991).) While there exists a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans. (Carillo, H., and Lipton, D., SIAM J Applied Math 48:1073 (1988).) Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in O'Guide to Huge Computers, O' Martin J. Bishop, ed., Academic Press, San Diego, (1994), and Carillo, H., and Lipton, D., SIAM J Applied Math 48:1073 (1988). Methods for aligning polynucleotides or polypeptides are codified in computer programs, including the GCG program package (Devereux, J., et al., Nucleic Acids Research (1984) 12(1):387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F. et al., J. Molec. Biol. 215:403 (1990)), Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711 (using the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981).))

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the VEGF2 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. The query sequence may be an entire sequence SEQ ID NO:1, the ORF (open reading frame), or any fragment specified as described herein.

As a practical matter, whether any particular nucleic acid molecule or polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence of the present invention can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and

a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. (1990) 6:237-245). In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by converting U's to T's. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of the present invention. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score.

For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In

another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, (indels) or substituted with another amino acid. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequences shown in Table 1 or to the amino acid sequence encoded by deposited DNA clone can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. (1990) 6:237-245). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1,

Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity

calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

### *VEGF-2 Polypeptides*

The present invention further relates to polypeptides which have the deduced amino acid sequence of FIGS. 1 or 2, or which has the amino acid sequence encoded by the deposited cDNAs, as well as fragments, analogs, and derivatives of such polypeptides.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of FIGS. 1 or 2 or that encoded by the deposited cDNA, means a polypeptide which retains the conserved motif of VEGF proteins as shown in FIG. 3 and essentially the same biological function or activity.

In the present invention, a "polypeptide fragment" refers to a short amino acid sequence contained in SEQ ID NO:2 or encoded by the cDNA contained in the deposited clone. Protein fragments may be "free-standing," or comprised within a larger polypeptide of which the fragment forms a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments from about amino acid number 1-20, 21-40, 41-60, 61-80, 81-100, 102-120, 121-140, 141-160, 161-180, 181-200, 201-220, 221-240, 241-260, 261-280, or 281 to the end of the coding region. Moreover, polypeptide fragments can be about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 amino acids in length. In this context "about" includes the particularly recited ranges, larger or smaller by several (5, 4, 3, 2, or 1) amino acids, at either extreme or at both extremes.

Preferred polypeptide fragments include the secreted VEGF2 protein as well as the mature form. Further preferred polypeptide fragments include the

secreted VEGF2 protein or the mature form having a continuous series of deleted residues from the amino or the carboxy terminus, or both. For example, any number of amino acids, ranging from 1-60, can be deleted from the amino terminus of either the secreted VEGF2 polypeptide or the mature form. Similarly, any number of amino acids, ranging from 1-30, can be deleted from the carboxy terminus of the secreted VEGF2 protein or mature form. Furthermore, any combination of the above amino and carboxy terminus deletions are preferred. Similarly, polynucleotide fragments encoding these VEGF2 polypeptide fragments are also preferred.

Also preferred are VEGF2 polypeptide and polynucleotide fragments characterized by structural or functional domains, such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Polypeptide fragments of SEQ ID NO:2 falling within conserved domains are specifically contemplated by the present invention. (See Figure 2.) Moreover, polynucleotide fragments encoding these domains are also contemplated.

Other preferred fragments are biologically active VEGF2 fragments. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of the VEGF2 polypeptide. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity.

The polypeptides of the present invention may be recombinant polypeptides, natural polypeptides, or synthetic polypeptides, preferably recombinant polypeptides.

It will be recognized in the art that some amino acid sequences of the VEGF2 polypeptide can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should



be remembered that there will be critical areas on the protein which determine activity.

Thus, the invention further includes variations of the VEGF2 polypeptide which show substantial VEGF2 polypeptide activity or which include regions of VEGF2 protein such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990).

Thus, the fragments, derivatives, or analogs of the polypeptides of FIGS. 1 or 2, or that encoded by the deposited cDNAs may be: (I) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code; or (ii) one in which one or more of the amino acid residues includes a substituent group; or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol); or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence; or (v) one in which comprises fewer amino acid residues shown in SEQ ID NOS: 2 or 4, and retains the conserved motif and yet still retains activity characteristics of the VEGF family of polypeptides. Such fragments, derivatives, and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Of particular interest are substitutions of charged amino acids with another charged amino acid and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the VEGF2 protein. The prevention of aggregation is highly desirable.

Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al.*, *Clin. Exp. Immunol.* 2:331-340 (1967); Robbins *et al.*, *Diabetes* 36:838-845 (1987); Cleland *et al.* *Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al.*, *Nature* 361:266-268 (1993) describes certain mutations resulting in selective binding of TNF- $\alpha$  to only one of the two known types of TNF receptors. Thus, the VEGF2 of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

**TABLE 1. Conservative Amino Acid Substitutions**

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Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

Of course, the number of amino acid substitutions a skilled artisan would make depends on many factors, including those described above. Generally speaking, the number of substitutions for any given VEGF-2 polypeptide will not be more than 50, 40, 30, 25, 20, 15, 10, 5 or 3.

Amino acids in the VEGF2 protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro*, or *in vitro* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al.*, *J. Mol. Biol.* 224:899-904 (1992) and de Vos *et al.* *Science* 255:306-312 (1992)).

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptides of SEQ ID NOS:2 and 4 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptides of SEQ ID NOS:2 and 4, and more preferably at least 90% similarity (more preferably at least 95% identity) to the polypeptides of SEQ ID NOS:2 and 4, and still more preferably at least 95% similarity (still more preferably at least 90% identity) to the polypeptides of SEQ ID NOS:2 and 4 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

5 The polypeptides of the present invention include the polypeptide encoded by the deposited cDNA including the leader; the mature polypeptide encoded by the deposited the cDNA minus the leader (i.e., the mature protein); a polypeptide comprising amino acids about - 23 to about 396 in SEQ ID NO:2; a polypeptide comprising amino acids about - 22 to about 396 in SEQ ID NO:2; a polypeptide comprising amino acids about 1 to about 396 in SEQ ID NO:2; as well as polypeptides which are at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to the polypeptides described above and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

#### Fusion Proteins

Any VEGF2 polypeptide can be used to generate fusion proteins. For example, the VEGF2 polypeptide, when fused to a second protein, can be used as an antigenic tag. Antibodies raised against the VEGF2 polypeptide can be used to indirectly detect the second protein by binding to the VEGF2. Moreover, because secreted proteins target cellular locations based on trafficking signals, the VEGF2 polypeptides can be used as a targeting molecule once fused to other proteins.

20 Examples of domains that can be fused to VEGF2 polypeptides include not only heterologous signal sequences, but also other heterologous functional regions. The fusion does not necessarily need to be direct, but may occur through linker sequences.

25 Moreover, fusion proteins may also be engineered to improve characteristics of the VEGF2 polypeptide. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the VEGF2 polypeptide to improve stability and persistence during purification from the host cell or subsequent handling and storage. Also, peptide moieties may be added to the VEGF2 polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the VEGF2 polypeptide. The addition of

peptide moieties to facilitate handling of polypeptides are familiar and routine techniques in the art.

Moreover, VEGF2 polypeptides, including fragments, and specifically epitopes, can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life in vivo. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP A 394,827; Traunecker et al., Nature 331:84-86 (1988).) Fusion proteins having disulfide-linked dimeric structures (due to the IgG) can also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis et al., J. Biochem. 270:3958-3964 (1995).)

Similarly, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP-A 0232 262.) Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. (See, D. Bennett et al., J. Molecular Recognition 8:52-58 (1995); K. Johanson et al., J. Biol. Chem. 270:9459-9471 (1995).)

Moreover, the VEGF2 polypeptides can be fused to marker sequences, such as a peptide which facilitates purification of VEGF2. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially

available. As described in Gentz et al., Proc. Natl. Acad. Sci. USA 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Another peptide tag useful for purification, the "HA" tag, corresponds to an epitope derived from the influenza hemagglutinin protein. (Wilson et al., Cell 37:767 (1984).)

Thus, any of these above fusions can be engineered using the VEGF2 polynucleotides or the polypeptides.

### ***Biological Activities of VEGF2***

VEGF2 polynucleotides and polypeptides can be used in assays to test for one or more biological activities. If VEGF2 polynucleotides and polypeptides do exhibit activity in a particular assay, it is likely that VEGF2 may be involved in the diseases associated with the biological activity. Therefore, VEGF2 could be used to treat the associated disease.

### ***Immune Activity***

VEGF2 polypeptides or polynucleotides may be useful in treating deficiencies or disorders of the immune system, by activating or inhibiting the proliferation, differentiation, or mobilization (chemotaxis) of immune cells. Immune cells develop through a process called hematopoiesis, producing myeloid (platelets, red blood cells, neutrophils, and macrophages) and lymphoid (B and T lymphocytes) cells from pluripotent stem cells. The etiology of these immune deficiencies or disorders may be genetic, somatic, such as cancer or some autoimmune disorders, acquired (e.g., by chemotherapy or toxins), or infectious. Moreover, VEGF2 polynucleotides or polypeptides can be used as a marker or detector of a particular immune system disease or disorder.

VEGF2 polynucleotides or polypeptides may be useful in treating or detecting deficiencies or disorders of hematopoietic cells. VEGF2 polypeptides or polynucleotides could be used to increase differentiation and proliferation of hematopoietic cells, including the pluripotent stem cells, in an effort to treat those

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disorders associated with a decrease in certain (or many) types hematopoietic cells. Examples of immunologic deficiency syndromes include, but are not limited to: blood protein disorders (e.g. agammaglobulinemia, dysgammaglobulinemia), ataxia telangiectasia, common variable immunodeficiency, Digeorge Syndrome, HIV infection, HTLV-BLV infection, leukocyte adhesion deficiency syndrome, lymphopenia, phagocyte bactericidal dysfunction, severe combined immunodeficiency (SCIDs), Wiskott-Aldrich Disorder, anemia, thrombocytopenia, or hemoglobinuria.

Moreover, VEGF2 polypeptides or polynucleotides can also be used to modulate hemostatic (the stopping of bleeding) or thrombolytic activity (clot formation). For example, by increasing hemostatic or thrombolytic activity, VEGF2 polynucleotides or polypeptides could be used to treat blood coagulation disorders (e.g., afibrinogenemia, factor deficiencies), blood platelet disorders (e.g. thrombocytopenia), or wounds resulting from trauma, surgery, or other causes. Alternatively, VEGF2 polynucleotides or polypeptides that can decrease hemostatic or thrombolytic activity could be used to inhibit or dissolve clotting, important in the treatment of heart attacks (infarction), strokes, or scarring.

VEGF2 polynucleotides or polypeptides may also be useful in treating or detecting autoimmune disorders. Many autoimmune disorders result from inappropriate recognition of self as foreign material by immune cells. This inappropriate recognition results in an immune response leading to the destruction of the host tissue. Therefore, the administration of VEGF2 polypeptides or polynucleotides that can inhibit an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing autoimmune disorders.

Examples of autoimmune disorders that can be treated or detected by VEGF2 include, but are not limited to: Addison's Disease, hemolytic anemia, antiphospholipid syndrome, rheumatoid arthritis, dermatitis, allergic encephalomyelitis, glomerulonephritis, Goodpasture's Syndrome, Graves Disease, Multiple Sclerosis, Myasthenia Gravis, Neuritis, Ophthalmia, Bullous



Pemphigoid, Pemphigus, Polyendocrinopathies, Purpura, Reiter's Disease, Stiff-Man Syndrome, Autoimmune Thyroiditis, Systemic Lupus Erythematosus, Autoimmune Pulmonary Inflammation, Guillain-Barre Syndrome, insulin dependent diabetes mellitus, and autoimmune inflammatory eye disease.

Similarly, allergic reactions and conditions, such as asthma (particularly allergic asthma) or other respiratory problems, may also be treated by VEGF2 polypeptides or polynucleotides. Moreover, VEGF2 can be used to treat anaphylaxis, hypersensitivity to an antigenic molecule, or blood group incompatibility.

VEGF2 polynucleotides or polypeptides may also be used to treat and/or prevent organ rejection or graft-versus-host disease (GVHD). Organ rejection occurs by host immune cell destruction of the transplanted tissue through an immune response. Similarly, an immune response is also involved in GVHD, but, in this case, the foreign transplanted immune cells destroy the host tissues. The administration of VEGF2 polypeptides or polynucleotides that inhibits an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing organ rejection or GVHD.

Similarly, VEGF2 polypeptides or polynucleotides may also be used to modulate inflammation. For example, VEGF2 polypeptides or polynucleotides may inhibit the proliferation and differentiation of cells involved in an inflammatory response. These molecules can be used to treat inflammatory conditions, both chronic and acute conditions, including inflammation associated with infection (e.g., septic shock, sepsis, or systemic inflammatory response syndrome (SIRS)), ischemia-reperfusion injury, endotoxin lethality, arthritis, complement-mediated hyperacute rejection, nephritis, cytokine or chemokine induced lung injury, inflammatory bowel disease, Crohn's disease, or resulting from over production of cytokines (e.g., TNF or IL-1.)

### Hyperproliferative Disorders

VEGF2 polypeptides or polynucleotides can be used to treat or detect hyperproliferative disorders, including neoplasms. VEGF2 antagonist polypeptides or polynucleotides may inhibit the proliferation of the disorder through direct or indirect interactions. Alternatively, VEGF2 antagonist polypeptides or polynucleotides may proliferate other cells which can inhibit the hyperproliferative disorder.

For example, by increasing an immune response, particularly increasing antigenic qualities of the hyperproliferative disorder or by proliferating, differentiating, or mobilizing T-cells, hyperproliferative disorders can be treated. This immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, decreasing an immune response may also be a method of treating hyperproliferative disorders, such as a chemotherapeutic agent.

Examples of hyperproliferative disorders that can be treated or detected by VEGF2 antagonist polynucleotides or polypeptides include, but are not limited to neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic, and urogenital.

Similarly, other hyperproliferative disorders can also be treated or detected by VEGF2 antagonist polynucleotides or polypeptides. Examples of such hyperproliferative disorders include, but are not limited to: hypergammaglobulinemia, lymphoproliferative disorders, paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenström's Macroglobulinemia, Gaucher's Disease, histiocytosis, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

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### Infectious Disease

VEGF2 polypeptides or polynucleotides can be used to treat or detect infectious agents. For example, by increasing the immune response, particularly increasing the proliferation and differentiation of B and/or T cells, infectious diseases may be treated. The immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, VEGF2 polypeptides or polynucleotides may also directly inhibit the infectious agent, without necessarily eliciting an immune response.

Viruses are one example of an infectious agent that can cause disease or symptoms that can be treated or detected by VEGF2 polynucleotides or polypeptides. Examples of viruses, include, but are not limited to the following DNA and RNA viral families: Arbovirus, Adenoviridae, Arenaviridae, Arterivirus, Birnaviridae, Bunyaviridae, Caliciviridae, Circoviridae, Coronaviridae, Flaviviridae, Hepadnaviridae (Hepatitis), Herpesviridae (such as, Cytomegalovirus, Herpes Simplex, Herpes Zoster), Mononegavirus (e.g., Paramyxoviridae, Morbillivirus, Rhabdoviridae), Orthomyxoviridae (e.g., Influenza), Papovaviridae, Parvoviridae, Picornaviridae, Poxviridae (such as Smallpox or Vaccinia), Reoviridae (e.g., Rotavirus), Retroviridae (HTLV-I, HTLV-II, Lentivirus), and Togaviridae (e.g., Rubivirus). Viruses falling within these families can cause a variety of diseases or symptoms, including, but not limited to: arthritis, bronchiolitis, encephalitis, eye infections (e.g., conjunctivitis, keratitis), chronic fatigue syndrome, hepatitis (A, B, C, E, Chronic Active, Delta), meningitis, opportunistic infections (e.g., AIDS), pneumonia, Burkitt's Lymphoma, chickenpox, hemorrhagic fever, Measles, Mumps, Parainfluenza, Rabies, the common cold, Polio, leukemia, Rubella, sexually transmitted diseases, skin diseases (e.g., Kaposi's, warts), and viremia. VEGF2 polypeptides or polynucleotides can be used to treat or detect any of these symptoms or diseases.

Similarly, bacterial or fungal agents that can cause disease or symptoms and that can be treated or detected by VEGF2 polynucleotides or polypeptides include, but not limited to, the following Gram-Negative and Gram-positive

bacterial families and fungi: Actinomycetales (e.g., *Corynebacterium*, *Mycobacterium*, *Nocardia*), Aspergillosis, Bacillaceae (e.g., *Anthrax*, *Clostridium*), Bacteroidaceae, Blastomycosis, *Bordetella*, *Borrelia*, *Brucellosis*, *Candidiasis*, *Campylobacter*, *Coccidioidomycosis*, *Cryptococcosis*, *Dermatocycoses*, Enterobacteriaceae (*Klebsiella*, *Salmonella*, *Serratia*, *Yersinia*), *Erysipelothrix*, *Helicobacter*, *Legionellosis*, *Leptospirosis*, *Listeria*, *Mycoplasmatales*, *Neisseriaceae* (e.g., *Acinetobacter*, *Gonorrhea*, *Menigococcal*), *Pasteurellacea* Infections (e.g., *Actinobacillus*, *Haemophilus*, *Pasteurella*), *Pseudomonas*, *Rickettsiaceae*, *Chlamydiaceae*, *Syphilis*, and *Staphylococcal*. These bacterial or fungal families can cause the following diseases or symptoms, including, but not limited to: bacteremia, endocarditis, eye infections (conjunctivitis, tuberculosis, uveitis), gingivitis, opportunistic infections (e.g., AIDS related infections), paronychia, prosthesis-related infections, *Reiter's* Disease, respiratory tract infections, such as Whooping Cough or Empyema, sepsis, Lyme Disease, Cat-Scratch Disease, Dysentery, Paratyphoid Fever, food poisoning, Typhoid, pneumonia, *Gonorrhea*, meningitis, *Chlamydia*, *Syphilis*, *Diphtheria*, *Leprosy*, *Paratuberculosis*, *Tuberculosis*, *Lupus*, *Botulism*, gangrene, tetanus, impetigo, *Rheumatic Fever*, *Scarlet Fever*, sexually transmitted diseases, skin diseases (e.g., cellulitis, dermatocycoses), toxemia, urinary tract infections, wound infections. VEGF2 polypeptides or polynucleotides can be used to treat or detect any of these symptoms or diseases.

Moreover, parasitic agents causing disease or symptoms that can be treated or detected by VEGF2 polynucleotides or polypeptides include, but not limited to, the following families: *Amebiasis*, *Babesiosis*, *Coccidiosis*, *Cryptosporidiosis*, *Dientamoebiasis*, *Dourine*, *Ectoparasitic*, *Giardiasis*, *Helminthiasis*, *Leishmaniasis*, *Theileriasis*, *Toxoplasmosis*, *Trypanosomiasis*, and *Trichomonas*. These parasites can cause a variety of diseases or symptoms, including, but not limited to: *Scabies*, *Trombiculiasis*, eye infections, intestinal disease (e.g., dysentery, giardiasis), liver disease, lung disease, opportunistic infections (e.g., AIDS related), *Malaria*, pregnancy complications, and

toxoplasmosis. VEGF2 polypeptides or polynucleotides can be used to treat or detect any of these symptoms or diseases.

Preferably, treatment using VEGF2 polypeptides or polynucleotides could either be by administering an effective amount of VEGF2 polypeptide to the patient, or by removing cells from the patient, supplying the cells with VEGF2 polynucleotide, and returning the engineered cells to the patient (ex vivo therapy). Moreover, the VEGF2 polypeptide or polynucleotide can be used as an antigen in a vaccine to raise an immune response against infectious disease.

### Regeneration

VEGF2 polynucleotides or polypeptides can be used to differentiate, proliferate, and attract cells, leading to the regeneration of tissues. (See, Science 276:59-87 (1997).) The regeneration of tissues could be used to repair, replace, or protect tissue damaged by congenital defects, trauma (wounds, burns, incisions, or ulcers), age, disease (e.g. osteoporosis, osteoarthritis, periodontal disease, liver failure), surgery, including cosmetic plastic surgery, fibrosis, reperfusion injury, or systemic cytokine damage.

Tissues that could be regenerated using the present invention include organs (e.g., pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac), vascular (including vascular endothelium), lymphatic (including lymphatic endothelium), nervous, hematopoietic, and skeletal (bone, cartilage, tendon, and ligament) tissue. Preferably, regeneration occurs without or decreased scarring. Regeneration also may include angiogenesis.

Moreover, VEGF2 polynucleotides or polypeptides may increase regeneration of tissues difficult to heal. For example, increased tendon/ligament regeneration would quicken recovery time after damage. VEGF2 polynucleotides or polypeptides of the present invention could also be used prophylactically in an effort to avoid damage. Specific diseases that could be treated include of tendinitis, carpal tunnel syndrome, and other tendon or ligament defects. A further

example of tissue regeneration of non-healing wounds includes pressure ulcers, ulcers associated with vascular insufficiency, surgical, and traumatic wounds.

Similarly, nerve and brain tissue could also be regenerated by using VEGF2 polynucleotides or polypeptides to proliferate and differentiate nerve cells. Diseases that could be treated using this method include central and peripheral nervous system diseases, neuropathies, or mechanical and traumatic disorders (e.g., spinal cord disorders, head trauma, cerebrovascular disease, and stroke). Specifically, diseases associated with peripheral nerve injuries, peripheral neuropathy (e.g., resulting from chemotherapy or other medical therapies), localized neuropathies, and central nervous system diseases (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome), could all be treated using the VEGF2 polynucleotides or polypeptides.

#### Chemotaxis

VEGF2 polynucleotides or polypeptides may have chemotaxis activity. A chemotactic molecule attracts or mobilizes cells (e.g., monocytes, fibroblasts, neutrophils, T-cells, mast cells, eosinophils, epithelial and/or endothelial cells) to a particular site in the body, such as inflammation, infection, or site of hyperproliferation. The mobilized cells can then fight off and/or heal the particular trauma or abnormality.

VEGF2 polynucleotides or polypeptides may increase chemotactic activity of particular cells. These chemotactic molecules can then be used to treat inflammation, infection, hyperproliferative disorders, or any immune system disorder by increasing the number of cells targeted to a particular location in the body. For example, chemotactic molecules can be used to treat wounds and other trauma to tissues by attracting immune cells to the injured location. As a chemotactic molecule, VEGF2 could also attract fibroblasts, which can be used to treat wounds.

It is also contemplated that VEGF2 polynucleotides or polypeptides may inhibit chemotactic activity. These molecules could also be used to treat disorders. Thus, VEGF2 polynucleotides or polypeptides could be used as an inhibitor of chemotaxis.

### Binding Activity

VEGF2 polypeptides may be used to screen for molecules that bind to VEGF2 or for molecules to which VEGF2 binds. The binding of VEGF2 and the molecule may activate (agonist), increase, inhibit (antagonist), or decrease activity of the VEGF2 or the molecule bound. Examples of such molecules include antibodies, oligonucleotides, proteins (e.g., receptors), or small molecules.

Preferably, the molecule is closely related to the natural ligand of VEGF2, e.g., a fragment of the ligand, or a natural substrate, a ligand, a structural or functional mimetic. (See, Coligan et al., Current Protocols in Immunology 1(2):Chapter 5 (1991).) Similarly, the molecule can be closely related to the natural receptor to which VEGF2 binds (i.e., Flt-4), or at least, a fragment of the receptor capable of being bound by VEGF2 (e.g., active site). In either case, the molecule can be rationally designed using known techniques.

Preferably, the screening for these molecules involves producing appropriate cells which express VEGF2, either as a secreted protein or on the cell membrane. Preferred cells include cells from mammals, yeast, *Drosophila*, or *E. coli*. Cells expressing VEGF2 (or cell membrane containing the expressed polypeptide) are then preferably contacted with a test compound potentially containing the molecule to observe binding, stimulation, or inhibition of activity of either VEGF2 or the molecule.

The assay may simply test binding of a candidate compound to VEGF2, wherein binding is detected by a label, or in an assay involving competition with a labeled competitor. Further, the assay may test whether the candidate compound results in a signal generated by binding to VEGF2.

Alternatively, the assay can be carried out using cell-free preparations, polypeptide/molecule affixed to a solid support, chemical libraries, or natural product mixtures. The assay may also simply comprise the steps of mixing a candidate compound with a solution containing VEGF2, measuring VEGF2/molecule activity or binding, and comparing the VEGF2/molecule activity or binding to a standard.

Preferably, an ELISA assay can measure VEGF2 level or activity in a sample (e.g., biological sample) using a monoclonal or polyclonal antibody. The antibody can measure VEGF2 level or activity by either binding, directly or indirectly, to VEGF2 or by competing with VEGF2 for a substrate.

All of these above assays can be used as diagnostic or prognostic markers. The molecules discovered using these assays can be used to treat disease or to bring about a particular result in a patient (e.g., blood vessel growth) by activating or inhibiting the VEGF2/molecule. Moreover, the assays can discover agents which may inhibit or enhance the production of VEGF2 from suitably manipulated cells or tissues.

Therefore, the invention includes a method of identifying compounds which bind to VEGF2 comprising the steps of: (a) incubating a candidate binding compound with VEGF2; and (b) determining if binding has occurred. Moreover, the invention includes a method of identifying agonists/antagonists comprising the steps of: (a) incubating a candidate compound with VEGF2, (b) assaying a biological activity, and (b) determining if a biological activity of VEGF2 has been altered.

#### Other Activities

VEGF2 polypeptides or polynucleotides may also increase or decrease the differentiation or proliferation of embryonic stem cells, besides, as discussed above, hematopoietic lineage.

VEGF2 polypeptides or polynucleotides may also be used to modulate mammalian characteristics, such as body height, weight, hair color, eye color, skin,



percentage of adipose tissue, pigmentation, size, and shape (e.g., cosmetic surgery). Similarly, VEGF2 polypeptides or polynucleotides may be used to modulate mammalian metabolism affecting catabolism, anabolism, processing, utilization, and storage of energy.

5 VEGF2 polypeptides or polynucleotides may be used to change a mammal's mental state or physical state by influencing biorhythms, cardiac rhythms, depression (including depressive disorders), tendency for violence, tolerance for pain, reproductive capabilities (preferably by Activin or Inhibin-like activity), hormonal or endocrine levels, appetite, libido, memory, stress, or other cognitive qualities.

VEGF2 polypeptides or polynucleotides may also be used as a food additive or preservative, such as to increase or decrease storage capabilities, fat content, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional components.

#### *Vectors and Host Cells*

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of VEGF2 polypeptides or peptides by recombinant techniques.

20 Host cells are genetically engineered (transduced, transformed, or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants, or amplifying the VEGF2 genes of the invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the skilled artisan.

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5 The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide sequence may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other plasmid or vector may be used so long as it is replicable and viable in the host.

10 The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

15 The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli* *lac* or *trp*, the phage lambda  $P_L$  promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

20 In addition, the expression vectors preferably contain at least one selectable marker gene to provide a phenotypic trait for selection of transformed host cells. Such markers include dihydrofolate reductase (DHFR) or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance for culturing in *E. coli* and other bacteria.

25 The vector containing the appropriate DNA sequence as herein above described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the

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protein. Representative examples of appropriate hosts, include but are not limited to: bacterial cells, such as *E. coli*, *Salmonella typhimurium*, and *Streptomyces*; fungal cells, such as yeast; insect cells, such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO, COS, and Bowes melanoma; and plant cells. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example - bacterial: pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

In addition to the use of expression vectors in the practice of the present invention, the present invention further includes novel expression vectors comprising operator and promoter elements operatively linked to nucleotide sequences encoding a protein of interest. One example of such a vector is pHE4a which is described in detail below.

As summarized in Figures 28 and 29, components of the pHE4a vector (SEQ ID NO:16) include: 1) a neomycinphosphotransferase gene as a selection marker, 2) an *E. coli* origin of replication, 3) a T5 phage promoter sequence, 4) two *lac* operator sequences, 5) a Shine-Delgarno sequence, 6) the lactose

operon repressor gene (*lacIq*) and 7) a multiple cloning site linker region. The origin of replication (*oriC*) is derived from pUC19 (LTI, Gaithersburg, MD). The promoter sequence and operator sequences were made synthetically. Synthetic production of nucleic acid sequences is well known in the art. CLONTECH 95/96 Catalog, pages 215-216, CLONTECH, 1020 East Meadow Circle, Palo Alto, CA 94303. The pHE4a vector was deposited with the ATCC on \_\_\_\_, 1998, and given accession number \_\_\_\_.

A nucleotide sequence encoding VEGF-2 (SEQ ID NO:1), is operatively linked to the promoter and operator of pHE4a by restricting the vector with *NdeI* and either *XbaI*, *BamHI*, *XhoI*, or *Asp718*, and isolating the larger fragment (the multiple cloning site region is about 310 nucleotides) on a gel. The nucleotide sequence encoding VEGF-2 (SEQ ID NO:1) having the appropriate restriction sites is generated, for example, according to the PCR protocol described in Example 1, using PCR primers having restriction sites for *NdeI* (as the 5' primer) and either *XbaI*, *BamHI*, *XhoI*, or *Asp718* (as the 3' primer). The PCR insert is gel purified and restricted with compatible enzymes. The insert and vector are ligated according to standard protocols.

As noted above, the pHE4a vector contains a *lacIq* gene. *LacIq* is an allele of the *lacI* gene which confers tight regulation of the *lac* operator. Amann, E. *et al.*, *Gene* 69:301-315 (1988); Stark, M., *Gene* 51:255-267 (1987). The *lacIq* gene encodes a repressor protein which binds to *lac* operator sequences and blocks transcription of down-stream (*i.e.*, 3') sequences. However, the *lacIq* gene product dissociates from the *lac* operator in the presence of either lactose or certain lactose analogs, *e.g.*, isopropyl B-D-thiogalactopyranoside (IPTG). VEGF-2 thus is not produced in appreciable quantities in uninduced host cells containing the pHE4a vector. Induction of these host cells by the addition of an agent such as IPTG, however, results in the expression of the VEGF-2 coding sequence.

The promoter/operator sequences of the pHE4a vector (SEQ ID NO:17) comprise a T5 phage promoter and two *lac* operator sequences. One operator is located 5' to the transcriptional start site and the other is located 3' to the same

site. These operators, when present in combination with the *lacIq* gene product, confer tight repression of down-stream sequences in the absence of a *lac* operon inducer, e.g., IPTG. Expression of operatively linked sequences located down-stream from the *lac* operators may be induced by the addition of a *lac* operon inducer, such as IPTG. Binding of a *lac* inducer to the *lacIq* proteins results in their release from the *lac* operator sequences and the initiation of transcription of operatively linked sequences. *Lac* operon regulation of gene expression is reviewed in Devlin, T., TEXTBOOK OF BIOCHEMISTRY WITH CLINICAL CORRELATIONS, 4th Edition (1997), pages 802-807.

The pHE4 series of vectors contain all of the components of the pHE4a vector except for the VEGF-2 coding sequence. Features of the pHE4a vectors include optimized synthetic T5 phage promoter, *lac* operator, and Shine-Delagarno sequences. Further, these sequences are also optimally spaced so that expression of an inserted gene may be tightly regulated and high level of expression occurs upon induction.

Among known bacterial promoters suitable for use in the production of proteins of the present invention include the *E. coli lacI* and *lacZ* promoters, the T3 and T7 promoters, the *gpt* promoter, the lambda PR and PL promoters and the *trp* promoter. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous Sarcoma Virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter.

The pHE4a vector also contains a Shine-Delgarno sequence 5' to the AUG initiation codon. Shine-Delgarno sequences are short sequences generally located about 10 nucleotides up-stream (i.e., 5') from the AUG initiation codon. These sequences essentially direct prokaryotic ribosomes to the AUG initiation codon.

Thus, the present invention is also directed to expression vector useful for the production of the proteins of the present invention. This aspect of the invention is exemplified by the pHE4a vector (SEQ ID NO:16).

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include *lacI*, *lacZ*, T3, T7, *gpt*,  $\lambda$  P<sub>R</sub>, P<sub>L</sub> and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described construct. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, electroporation, transduction, infection, or other methods (Davis, L., *et al.*, *Basic Methods in Molecular Biology* (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989), the disclosure of which is hereby incorporated by reference.

Transcription of a DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the

SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, *e.g.*, stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden)

and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is derepressed by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, well known to those skilled in the art, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell* 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptides can be recovered and purified from recombinant cell cultures by methods used heretofore, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. It is preferred to have low concentrations (approximately 0.1-0.5mM) of calcium ion present during purification (Price *et al.*, *J. Biol. Chem.*



244:917 (1969)). Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

#### *Therapeutic Uses*

The VEGF-2 polypeptide of the present invention is a potent mitogen for vascular and lymphatic endothelial cells. As shown in Figures 12 and 13, the VEGF2 polypeptide of SEQ ID NO:2, minus the initial 46 amino acids, is a potent mitogen for vascular endothelial cells and stimulates their growth and proliferation. The results of a Northern blot analysis performed for the VEGF2 nucleic acid sequence encoding this polypeptide wherein 20  $\mu$ g of RNA from several human tissues were probed with  $^{32}$ P-VEGF2, illustrates that this protein is actively expressed in the heart and lung which is further evidence of mitogenic activity.

Accordingly, VEGF2, or biologically active portions thereof, may be employed to treat vascular trauma by promoting angiogenesis. For example, to stimulate the growth of transplanted tissue where coronary bypass surgery is performed. VEGF2, or biologically active portions thereof, may also be employed to promote wound healing, particularly to re-vascularize damaged tissues or stimulate collateral blood flow during ischemia and where new capillary angiogenesis is desired. VEGF2, or biologically active portions thereof, may be employed to treat full-thickness wounds such as dermal ulcers, including pressure

sores, venous ulcers, and diabetic ulcers. In addition, VEGF2, or biologically active portions thereof, may be employed to treat full-thickness burns and injuries where a skin graft or flap is used to repair such burns and injuries. VEGF2, or biologically active portions thereof, may also be employed for use in plastic surgery, for example, for the repair of lacerations, burns, or other trauma. In addition, VEGF-2 can be used to promote healing of wounds and injuries to the eye as well as to treat eye diseases.

Along these same lines, VEGF2, or biologically active portions thereof, may also be employed to induce the growth of damaged bone, periodontium or ligament tissue. VEGF2, or biologically active portions thereof, may also be employed for regenerating supporting tissues of the teeth, including cementum and periodontal ligament, that have been damaged by, *e.g.*, periodental disease or trauma.

Since angiogenesis is important in keeping wounds clean and non-infected, VEGF2, or biologically active portions thereof, may be employed in association with surgery and following the repair of incisions and cuts. VEGF2, or biologically active portions thereof, may also be employed for the treatment of abdominal wounds where there is a high risk of infection.

VEGF2, or biologically active portions thereof, may be employed for the promotion of endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted or synthetic material, VEGF2, or biologically active portions thereof, can be applied to the surface of the graft or at the junction to promote the growth of vascular endothelial cells. VEGF2, or biologically active portions thereof, may also be employed to repair damage of myocardial tissue as a result of myocardial infarction. VEGF2, or biologically active portions thereof, may also be employed to repair the cardiac vascular system after ischemia. VEGF2, or biologically active portions thereof, may also be employed to treat damaged vascular tissue as a result of coronary artery disease and peripheral and CNS vascular disease.

VEGF2, or biologically active portions thereof, may also be employed to coat artificial prostheses or natural organs which are to be transplanted in the body to minimize rejection of the transplanted material and to stimulate vascularization of the transplanted materials.

5 VEGF2, or biologically active portions thereof, may also be employed for vascular tissue repair of injuries resulting from trauma, for example, that occurring during arteriosclerosis and required following balloon angioplasty where vascular tissues are damaged.

VEGF2, or biologically active portions thereof, may also be used to treat peripheral arterial disease. Accordingly, in a further aspect, there is provided a process for utilizing VEGF2 polypeptides to treat peripheral arterial disease. Preferably, a VEGF-2 polypeptide is administered to an individual for the purpose of alleviating or treating peripheral arterial disease. Suitable doses, formulations, and administration routes are described below.

VEGF2, or biologically active portions thereof, may also to promote the endothelial function of lymphatic tissues and vessels, such as to treat the loss of lymphatic vessels, occlusions of lymphatic vessels, and lymphangiomas. VEGF-2 may also be used to stimulate lymphocyte production.

20 VEGF2, or biologically active portions thereof, may also be used to treat hemangioma in newborns. Accordingly, in a further aspect, there is provided a process for utilizing VEGF2 polypeptides to treat hemangioma in newborns. Preferably, a VEGF-2 polypeptide is administered to an individual for the purpose of alleviating or treating hemangioma in newborns. Suitable doses, formulations, and administration routes are described below.

25 VEGF2, or biologically active portions thereof, may also be used to prevent or treat abnormal retinal development in premature newborns. Accordingly, in a further aspect, there is provided a process for utilizing VEGF2 polypeptides to treat abnormal retinal development in premature newborns. Preferably, a VEGF-2 polypeptide is administered to an individual for the purpose

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of alleviating or treating abnormal retinal development in premature newborns. Suitable doses, formulations, and administration routes are described below.

VEGF2, or biologically active portions thereof, may be used to treat primary (idiopathic) lymphademas, including Milroy's disease and Lymphedema praecox. Accordingly, in a further aspect, there is provided a process for utilizing VEGF2 polypeptides to treat primary (idiopathic) lymphademas, including Milroy's disease and Lymphedema praecox. Preferably, a VEGF-2 polypeptide is administered to an individual for the purpose of alleviating or treating primary (idiopathic) lymphademas, including Milroy's disease and Lymphedema praecox. VEGF-2 or biologically active portions thereof, may also be used to treat edema as well as to effect blood pressure in an animal. Suitable doses, formulations, and administration routes are described below.

VEGF2, or biologically active portions thereof, may also be used to treat secondary (obstructive) lymphademas including those that result from (i) the removal of lymph nodes and vessels, (ii) radiotherapy and surgery in the treatment of cancer, and (iii) trauma and infection. Accordingly, in a further aspect, there is provided a process for utilizing VEGF2 polypeptides to treat secondary (obstructive) lymphademas including those that result from (i) the removal of lymph nodes and vessels, (ii) radiotherapy and surgery in the treatment of cancer, and (iii) trauma and infection. Preferably, a VEGF-2 polypeptide is administered to an individual for the purpose of secondary (obstructive) lymphademas including those that result from (i) the removal of lymph nodes and vessels, (ii) radiotherapy and surgery in the treatment of cancer, and (iii) trauma and infection. Suitable doses, formulations, and administration routes are described below.

VEGF2, or biologically active portions thereof, may also be used to treat Kaposi's Sarcoma. Accordingly, in a further aspect, there is provided a process for utilizing VEGF2 polypeptides to treat Kaposi's Sarcoma. Preferably, a VEGF-2 polypeptide is administered to an individual for the purpose of alleviating or treating Kaposi's Sarcoma. Suitable doses, formulations, and administration routes are described below.

VEGF-2 antagonists can be used to treat cancer by inhibiting the angiogenesis necessary to support cancer and tumor growth.

### *Gene Therapy Methods*

Another aspect of the present invention is to gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA and antisense DNA or RNA) sequences into an animal to achieve expression of the VEGF-2 polypeptide of the present invention. This method requires polynucleotide which codes for a VEGF-2 polypeptide operatively linked to a promoter and any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques are known in the art, see, for example, WO90/11092, which is herein incorporated by reference.

As discussed in more detail below, the VEGF-2 polynucleotide constructs can be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, and the like). The VEGF-2 polynucleotide constructs are delivered in a pharmaceutically acceptable liquid or aqueous carrier.

The term "naked" polynucleotide, DNA or RNA refers to sequences that are free from any delivery vehicle that acts to assist, promote or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the VEGF-2 polynucleotides can also be delivered in liposome formulations and lipofectin formulations and the like can be prepared by methods well known to those skilled in the art.

The VEGF-2 polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of VEGF-2 DNA. Unlike other gene therapies techniques, one major advantage of introducing

naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The VEGF-2 polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. *In vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked acid sequence injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05  $\mu\text{g/kg}$  body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration.

5 The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked VEGF-2 DNA constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

As is evidenced by Example 18, naked VEGF-2 nucleic acid sequences can be administered *in vivo* results in the successful expression of VEGF-2 polypeptide in the femoral arteries of rabbits.

### *Nucleic Acid Utilities*

VEGF2 nucleic acid sequences and VEGF2 polypeptides may also be employed for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors, and for the production of diagnostics and therapeutics to treat human disease. For example, VEGF2 may be employed for *in vitro* culturing of vascular endothelial cells, where it is added to the conditional medium in a concentration from 10 pg/ml to 10 ng/ml.

20 Fragments of the full length VEGF2 gene may be used as a hybridization probe for a cDNA library to isolate other genes which have a high sequence similarity to the gene or similar biological activity. Probes of this type generally have at least 50 base pairs, although they may have a greater number of bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete VEGF2 gene including regulatory and promotor regions, exons, and introns. An example of a  
25 screen comprises isolating the coding region of the VEGF2 gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

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5 This invention provides methods for identification of VEGF2 receptors. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan *et al.*, *Current Protocols in Immun.*, 1(2), Chapter 5, (1991)). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to VEGF2, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to VEGF2. Transfected cells which are grown on glass slides are exposed to labeled VEGF2. VEGF2 can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and retransfected using an iterative sub-pooling and rescreening process, eventually yielding a single clone that encodes the putative receptor.

15 As an alternative approach for receptor identification, labeled VEGF2 can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray film. The labeled complex containing VEGF2 is then excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

### ***VEGF-2 Agonist and Antagonists***

25 This invention is also related to a method of screening compounds to identify those which are VEGF2 agonists or antagonists. An example of such a method takes advantage of the ability of VEGF2 to significantly stimulate the proliferation of human endothelial cells in the presence of the comitogen Con A. Endothelial cells are obtained and cultured in 96-well flat-bottomed culture plates (Costar, Cambridge, MA) in a reaction mixture supplemented with Con-A

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(Calbiochem, La Jolla, CA). Con-A, polypeptides of the present invention and the compound to be screened are added. After incubation at 37°C, cultures are pulsed with 1  $\mu$ Ci of  $^3$ [H]thymidine (5 Ci/mmol; 1 Ci = 37 BGq; NEN) for a sufficient time to incorporate the  $^3$ [H] and harvested onto glass fiber filters (Cambridge Technology, Watertown, MA). Mean  $^3$ [H]-thymidine incorporation (cpm) of triplicate cultures is determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA). Significant  $^3$ [H]thymidine incorporation, as compared to a control assay where the compound is excluded, indicates stimulation of endothelial cell proliferation.

To assay for antagonists, the assay described above is performed and the ability of the compound to inhibit  $^3$ [H]thymidine incorporation in the presence of VEGF2 indicates that the compound is an antagonist to VEGF2. Alternatively, VEGF2 antagonists may be detected by combining VEGF2 and a potential antagonist with membrane-bound VEGF2 receptors or recombinant receptors under appropriate conditions for a competitive inhibition assay. VEGF2 can be labeled, such as by radioactivity, such that the number of VEGF2 molecules bound to the receptor can determine the effectiveness of the potential antagonist.

Alternatively, the response of a known second messenger system following interaction of VEGF2 and receptor would be measured and compared in the presence or absence of the compound. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis. In another method, a mammalian cell or membrane preparation expressing the VEGF2 receptor is incubated with labeled VEGF2 in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured.

Potential VEGF2 antagonists include an antibody, or in some cases, an oligonucleotide, which bind to the polypeptide and effectively eliminate VEGF2 function. Alternatively, a potential antagonist may be a closely related protein which binds to VEGF2 receptors, however, they are inactive forms of the polypeptide and thereby prevent the action of VEGF2. Examples of these

antagonists include a negative dominant mutant of the VEGF2 polypeptide, for example, one chain of the hetero-dimeric form of VEGF2 may be dominant and may be mutated such that biological activity is not retained. An example of a negative dominant mutant includes truncated versions of a dimeric VEGF2 which is capable of interacting with another dimer to form wild type VEGF2, however, the resulting homo-dimer is inactive and fails to exhibit characteristic VEGF activity.

Another potential VEGF2 antagonist is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee *et al.*, *Nucl. Acids Res.* 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science* 251:1360 (1991)), thereby preventing transcription and the production of VEGF2. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the VEGF2 polypeptide (Antisense - Okano, *J. Neurochem.* 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of VEGF2.

Potential VEGF2 antagonists also include small molecules which bind to and occupy the active site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

5 The antagonists may be employed to limit angiogenesis necessary for solid tumor metastasis. The identification of VEGF2 can be used for the generation of certain inhibitors of vascular endothelial growth factor. Since angiogenesis and neovascularization are essential steps in solid tumor growth, inhibition of angiogenic activity of the vascular endothelial growth factor is very useful to prevent the further growth, retard, or even regress solid tumors. Although the level of expression of VEGF2 is extremely low in normal tissues including breast, it can be found expressed at moderate levels in at least two breast tumor cell lines that are derived from malignant tumors. It is, therefore, possible that VEGF2 is involved in tumor angiogenesis and growth.

Gliomas are also a type of neoplasia which may be treated with the antagonists of the present invention.

The antagonists may also be used to treat chronic inflammation caused by increased vascular permeability. In addition to these disorders, the antagonists may also be employed to treat retinopathy associated with diabetes, rheumatoid arthritis and psoriasis.

The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, *e.g.*, as hereinafter described.

20 The VEGF2 polypeptides and agonists and antagonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide or agonist or antagonist, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

25 The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the

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pharmaceutical compositions may be employed in conjunction with other therapeutic compounds.

5 The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, intratumor, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the pharmaceutical compositions are administered in an amount of at least about 10  $\mu\text{g/kg}$  body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10  $\mu\text{g/kg}$  to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

10 The VEGF2 polypeptides, and agonists or antagonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptide *in vivo*, which is often referred to as "gene therapy."

15 Thus, for example, cells such as bone marrow cells may be engineered with a polynucleotide (DNA or RNA) encoding for the polypeptide *ex vivo*, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA  
20 encoding the polypeptide of the present invention.

25 Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo*, for example, by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding a polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may

be other than a retroviral particle, for example, an adenovirus, which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

5 Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

10 The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller *et al.*, *Biotechniques* 7:980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and  $\beta$ -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

20 The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; 25 heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the  $\beta$ -actin promoter; and human growth hormone

promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317,  $\psi$ -2,  $\psi$ -AM, PA12, T19-14X, VT-19-17-H2,  $\psi$ CRE,  $\psi$ CRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, *Human Gene Therapy* 1:5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and  $\text{CaPO}_4$  precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

This invention is also related to the use of the VEGF2 gene as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutations in VEGF2 nucleic acid sequences.

Individuals carrying mutations in the VEGF2 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, *Nature* 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding VEGF2 can be

used to identify and analyze VEGF2 mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled VEGF2 RNA or alternatively, radiolabeled VEGF2 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, *Science* 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton *et al.*, *PNAS, USA* 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of VEGF2 protein in various tissues since an over-expression of the proteins compared to normal control tissue samples may detect the presence of a disease or susceptibility to a disease, for example, abnormal cellular differentiation. Assays used to detect levels of VEGF2 protein in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-

binding assays, Western Blot analysis, ELISA assays and "sandwich" assay. An ELISA assay (Coligan *et al.*, *Current Protocols in Immunology* 1(2), Chapter 6, (1991)) initially comprises preparing an antibody specific to the VEGF2 antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or, in this example, a horseradish peroxidase enzyme. A sample is removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein, such as, bovine serum albumen. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any VEGF2 proteins attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to VEGF2. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of VEGF2 protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to VEGF2 are attached to a solid support. Polypeptides of the present invention are then labeled, for example, by radioactivity, and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of VEGF2 in the sample.

A "sandwich" assay is similar to an ELISA assay. In a "sandwich" assay VEGF2 is passed over a solid support and binds to antibody attached to a solid support. A second antibody is then bound to the VEGF2. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantified.



5 The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphism's) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

10 Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

15 PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

20 Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with probes from the cDNA as short as 50 or 60 base pairs. For a review of this technique, see Verma *et al.*, *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

25 Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with

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genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that cDNA sequence. Ultimately, complete sequencing of genes from several individuals is required to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

The present invention is further directed to inhibiting VEGF2 *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the mature polynucleotide sequence, which encodes for the polypeptide of the present invention, is used to design an antisense RNA oligonucleotide of from 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -- see Lee *et al.*, *Nucl. Acids Res.* 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.* *Science*, 251:1360 (1991), thereby preventing transcription and the production of VEGF2. The antisense RNA

oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of an mRNA molecule into the VEGF2 (antisense -- Okano, *J. Neurochem.* 56:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988)).

5 Alternatively, the oligonucleotides described above can be delivered to cells by procedures in the art such that the anti-sense RNA or DNA may be expressed *in vivo* to inhibit production of VEGF2 in the manner described above.

Antisense constructs to VEGF2, therefore, may inhibit the angiogenic activity of the VEGF2 and prevent the further growth or even regress solid tumors, since angiogenesis and neovascularization are essential steps in solid tumor growth. These antisense constructs may also be used to treat rheumatoid arthritis, psoriasis, diabetic retinopathy and Kaposi's sarcoma which are all characterized by abnormal angiogenesis.

10 The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

20 Antibodies generated against the polypeptide corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptide into an animal or by administering the polypeptide to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies binding the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

25 For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, *Nature* 256:495-497 (1975)), the

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trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, *Immunology Today* 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985), pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention.

Neutralization antibodies can be identified and applied to mask the vascular endothelial growth factor, and that has been shown in mice model systems against VEGF. VEGF2 can also be inactivated by certain dominant negative mutants within the gene itself. It is known that both PDGF $\alpha$  and  $\beta$  form either heterodimers or homodimers, and VEGF forms homodimers. Similar interaction between VEGF2 could be expected. These antibodies therefore may be used to block the angiogenic activity of VEGF2 and retard the growth of solid tumors. These antibodies may also be used to treat inflammation caused by the increased vascular permeability which results from the presence of VEGF2.

These antibodies may further be used in an immunoassay to detect the presence of tumors in certain individuals. Enzyme immunoassay can be performed from the blood sample of an individual. Elevated levels of VEGF2 can be considered diagnostic of cancer.

The present invention is also directed to antagonist/inhibitors of the polypeptides of the present invention. The antagonist/inhibitors are those which inhibit or eliminate the function of the polypeptide.

Thus, for example, antagonists bind to a polypeptide of the present invention and inhibit or eliminate its function. The antagonist, for example, could be an antibody against the polypeptide which binds to the polypeptide or, in some cases, an oligonucleotide. An example of an inhibitor is a small molecule which binds to and occupies the catalytic site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is

prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

Truncated versions of VEGF2 can also be produced that are capable of interacting with wild type VEGF2 to form dimers that fail to activate endothelial cell growth, therefore inactivating the endogenous VEGF2. Or, mutant forms of VEGF2 form dimers themselves and occupy the ligand binding domain of the proper tyrosine kinase receptors on the target cell surface, but fail to activate cell growth.

Alternatively, antagonists to the polypeptides of the present invention may be employed which bind to the receptors to which a polypeptide of the present invention normally binds. The antagonists may be closely related proteins such that they recognize and bind to the receptor sites of the natural protein, however, they are inactive forms of the natural protein and thereby prevent the action of VEGF2 since receptor sites are occupied. In these ways, the action of the VEGF2 is prevented and the antagonist/inhibitors may be used therapeutically as an anti-tumor drug by occupying the receptor sites of tumors which are recognized by VEGF2 or by inactivating VEGF2 itself. The antagonist/inhibitors may also be used to prevent inflammation due to the increased vascular permeability action of VEGF2. The antagonist/inhibitors may also be used to treat solid tumor growth, diabetic retinopathy, psoriasis and rheumatoid arthritis.

The antagonist/inhibitors may be employed in a composition with a pharmaceutically acceptable carrier, *e.g.*, as hereinabove described.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples, certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially

available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

5 "Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

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15 Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, *Nucleic Acids Res.* 8:4057 (1980).

20 "Oligonucleotides" refer to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands, which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

25 "Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989), p. 146). Unless otherwise provided, ligation may be

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accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5  $\mu$ g of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described by the method of Graham, F. and Van der Eb, A., *Virology* 52:456-457 (1973).

### Example 1

#### *Expression Pattern of VEGF2 in Human Tissues and Breast Cancer Cell Lines*

Northern blot analysis was carried out to examine the levels of expression of VEGF2 in human tissues and breast cancer cell lines in human tissues. Total cellular RNA samples were isolated with RNAzol™ B system (Biotecx Laboratories, Inc.). About 10  $\mu$ g of total RNA isolated from each breast tissue and cell line specified was separated on 1% agarose gel and blotted onto a nylon filter, (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). The labeling reaction was done according to the Stratagene Prime-It kit with 50 ng DNA fragment. The labeled DNA was purified with a Select-G-50 column from 5 Prime  $\rightarrow$  3 Prime, Inc (Boulder, CO). The filter was then hybridized with a radioactive labeled full length VEGF2 gene at 1,000,000 cpm/ml in 0.5 M NaPO<sub>4</sub> and 7% SDS overnight at 65°C. After washing twice at room temperature and twice at 60°C with 0.5 X SSC, 0.1% SDS, the filters were then exposed at -70°C overnight with an intensifying screen. A message of 1.6 Kd was observed in 2 breast cancer cell lines. FIG. 5, lane #4 represents a very tumorigenic cell line that is estrogen independent for growth.

Also, 10  $\mu$ g of total RNA from 10 human adult tissues were separated on an agarose gel and blotted onto a nylon filter. The filter was then hybridized with radioactively labeled VEGF2 probe in 7% SDS, 0.5 M NaPO<sub>4</sub>, pH 7.2; 1% BSA overnight at 65°C. Following washing in 0.2 X SSC at 65°C, the filter was exposed to film for 24 days at -70°C with intensifying screen. See FIG. 6.

## Example 2

### *Expression of the Truncated Form of VEGF2 (SEQ ID NO:4) by in vitro Transcription and Translation*

The VEGF2 cDNA was transcribed and translated *in vitro* to determine the size of the translatable polypeptide encoded by the truncated form of VEGF2 and a partial VEGF2 cDNA. The two inserts of VEGF2 in the pBluescript SK vector were amplified by PCR with three pairs of primers, 1) M13-reverse and forward primers; 2) M13-reverse primer and VEGF primer F4; and 3) M13-reverse primer and VEGF primer F5. The sequence of these primers are as follows.

M13-2 reverse primer:

5'-ATGCTTCCGGCTCGTATG-3' (SEQ ID NO: 9)

This sequence is located upstream of the 5' end of the VEGF2 cDNA insert in the pBluescript vector and is in an anti-sense orientation as the cDNA. A T3 promoter sequence is located between this primer and the VEGF2 cDNA.

M13-2 forward primer:

5'GGGTTTTCCAGTCACGAC-3' (SEQ ID NO: 10)

This sequence is located downstream of the 3' end of the VEGF2 cDNA insert in the pBluescript vector and is in an anti-sense orientation as the cDNA insert.

VEGF primer F4:

5'-CCACATGGTTCAGGAAAGACA-3' (SEQ ID NO: 11)

This sequence is located within the VEGF2 cDNA in an anti-sense orientation from bp 1259-1239, which is about 169 bp away from the 3' end of the stop codon and about 266 bp before the last nucleotide of the cDNA.

PCR reaction with all three pairs of primers produce amplified products with T3 promoter sequence in front of the cDNA insert. The first and third pairs of primers produce PCR products that encode the polypeptide of VEGF2 shown in SEQ ID NO:4. The second pair of primers produce PCR product that misses 36 amino acids coding sequence at the C-terminus of the VEGF2 polypeptide.



Approximately 0.5  $\mu$ g of PCR product from first pair of primers, 1  $\mu$ g from second pair of primers, 1  $\mu$ g from third pair of primers were used for *in vitro* transcription/translation. The *in vitro* transcription/translation reaction was performed in a 25  $\mu$ l of volume, using the T<sub>N</sub>T™ Coupled Reticulocyte Lysate Systems (Promega, CAT# L4950). Specifically, the reaction contains 12.5  $\mu$ l of T<sub>N</sub>T rabbit reticulocyte lysate 2  $\mu$ l of T<sub>N</sub>T reaction buffer, 1  $\mu$ l of T3 polymerase, 1  $\mu$ l of 1 mM amino acid mixtrue (minus methionine), 4  $\mu$ l of <sup>35</sup>S-methionine (>1000 Ci/mmol, 10 mCi/ml), 1  $\mu$ l of 40 U/ul; RNasin ribonuclease inhibitor, 0.5 or 1  $\mu$ g of PCR products. Nuclease-free H<sub>2</sub>O was added to bring the volume to 25  $\mu$ l. The reaction was incubated at 30°C for 2 hours. Five microliters of the reaction product was analyzed on a 4-20% gradient SDS-PAGE gel. After fixing in 25% isopropanol and 10% acetic acid, the gel was dried and exposed to an X-ray film overnight at 70°C.

As shown in FIG. 7, PCR products containing the truncated VEGF2 cDNA (*i.e.*, as depicted in SEQ ID NO:3) and the cDNA missing 266 bp in the 3' untranslated region (3'-UTR) produced the same length of translated products, whose molecular weights are estimated to be 38-40 dk (lanes 1 and 3). The cDNA missing all the 3'UTR and missing sequence encoding the C-terminal 36 amino acids was translated into a polypeptide with an estimated molecular weight of 36-38 kd (lane 2).

### Example 3

#### *Cloning and Expression of VEGF2 Using the Baculovirus Expression System*

The DNA sequence encoding the VEGF2 protein without 46 amino acids at the N-terminus, see ATCC No. 97149, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence TGT AAT ACG ACT CAC TAT AGG GAT CCC GCC ATG GAG GCC ACG GCT TAT GC (SEQ ID NO:12) and

contains a BamHI restriction enzyme site (in bold) and 17 nucleotide sequence complementary to the 5' sequence of VEGF2 (nt. 150-166).

The 3' primer has the sequence GATC TCT AGA TTA GCT CAT TTG TGG TCT (SEQ ID NO:13) and contains the cleavage site for the restriction enzyme XbaI and 18 nucleotides complementary to the 3' sequence of VEGF2, including the stop codon and 15 nt sequence before stop codon.

The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("GeneClean," BIO 101, Inc., La Jolla, CA). The fragment was then digested with the endonuclease BamHI and XbaI and then purified again on a 1% agarose gel. This fragment was ligated to pAcGP67A baculovirus transfer vector (Pharmingen) at the BamHI and XbaI sites. Through this ligation, VEGF2 cDNA was cloned in frame with the signal sequence of baculovirus gp67 gene and was located at the 3' end of the signal sequence in the vector. This is designated pAcGP67A-VEGF2.

To clone VEGF2 with the signal sequence of gp67 gene to the pRG1 vector for expression, VEGF2 with the signal sequence and some upstream sequence were excised from the pAcGP67A-VEGF2 plasmid at the Xho restriction endonuclease site located upstream of the VEGF2 cDNA and at the XbaI restriction endonuclease site by XhoI and XbaI restriction enzyme. This fragment was separated from the rest of vector on a 1% agarose gel and was purified using "GeneClean" kit. It was designated F2.

The PRG1 vector (modification of pVL941 vector) is used for the expression of the VEGF2 protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E., "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures," Texas Agricultural Experimental Station Bulletin No. 1555, (1987)). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamHI, SmaI, XbaI, BglII and Asp718. A site for restriction endonuclease XhoI is located upstream of BamHI site. The sequence between XhoI and BamHI is the

same as that in PAcGp67A (static on tape) vector. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from *E.coli* is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of cotransfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., *Virology* 170:31-39 (1989)).

The plasmid was digested with the restriction enzymes XbaI and XbaI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("GeneClean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. *E.coli* HB101 cells were then transformed and bacteria identified that contained the plasmid (pBac gp67-VEGF2) with the VEGF2 gene using the enzymes BamHI and XbaI. The sequence of the cloned fragment was confirmed by DNA sequencing.

5 µg of the plasmid pBac gp67-VEGF2 was cotransfected with 1.0 µg of a commercially available linearized baculovirus ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofectin method (Felgner et al. *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987)).

1 µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac gp67-VEGF2 were mixed in a sterile well of a microtiter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added dropwise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate was rocked back and forth to

mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith, *supra*. As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the virus was added to the cells, blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculovirus was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-gp67-VEGF2 at a multiplicity of infection (MOI) of 1. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5 µCi of <sup>35</sup>S-methionine and 5 µCi <sup>35</sup>S cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

Protein from the medium and cytoplasm of the Sf9 cells was analyzed by SDS-PAGE under non-reducing and reducing conditions. See Figures 8A and 8B, respectively. The medium was dialyzed against 50 mM MES, pH 5.8. Precipitates were obtained after dialysis and resuspended in 100 mM NaCitrate, pH 5.0. The

resuspended precipitate was analyzed again by SDS-PAGE and was stained with Coomassie Brilliant Blue. See Figure 9.

The medium supernatant was also diluted 1:10 in 50 mM MES, pH 5.8 and applied to an SP-650M column (1.0 x 6.6 cm, Toyopearl) at a flow rate of 1 ml/min. Protein was eluted with step gradients at 200, 300 and 500 mM NaCl. The VEGF2 was obtained using the elution at 500 mM. The eluate was analyzed by SDS-PAGE in the presence or absence of reducing agent,  $\beta$ -mercaptoethanol and stained by Coomassie Brilliant Blue. See Figure 10.

### Example 4

#### *Expression of Recombinant VEGF2 in COS Cells*

The expression of plasmid, VEGF2-HA is derived from a vector pcDNA1/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) *E. coli* replication origin, 4) CMV promoter followed by a polylinker region, an SV40 intron and polyadenylation site. A DNA fragment encoding the entire VEGF2 precursor and a HA tag fused in frame to its 3' end was cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (Wilson *et al.*, *Cell* 37:767 (1984)). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding VEGF2, ATCC No. 97149, was constructed by PCR using two primers: the 5' primer (CGC GGA TCC ATG ACT GTA CTC TAC CCA) (SEQ ID NO:14) contains a BamHI site followed by 18 nucleotides of VEGF2 coding sequence starting from the initiation codon; the 3' sequence (CGC TCT AGA TCA AGC GTA GTC TGG GAC GTC GTA TGG GTA CTC

GAG GCT CAT TTG TGG TCT 3') (SEQ ID NO:15) contains complementary sequences to an XbaI site, HA tag, XhoI site, and the last 15 nucleotides of the VEGF2 coding sequence (not including the stop codon). Therefore, the PCR product contains a BamHI site, coding sequence followed by an XhoI restriction endonuclease site and HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XbaI site. The PCR amplified DNA fragment and the vector, pcDNAI/Amp, were digested with BamHI and XbaI restriction enzyme and ligated. The ligation mixture was transformed into *E. coli* strain SURE (Stratagene Cloning Systems, La Jolla, CA 92037) the transformed culture was plated on ampicillin media plates and resistant colonies were selected. Plasmid DNA was isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant VEGF2, COS cells were transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Laboratory Press, (1989)). The expression of the VEGF2-HA protein was detected by radiolabelling and immunoprecipitation method (E. Harlow and D. Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, (1988)). Cells were labelled for 8 hours with <sup>35</sup>S-cysteine two days post transfection. Culture media was then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson *et al.*, *Cell* 37:767 (1984)). Both cell lysate and culture media were precipitated with an HA specific monoclonal antibody. Proteins precipitated were analyzed on 15% SDS-PAGE gels.

### Example 5

***The Effect of Partially Purified VEGF2 Protein on the Growth of Vascular Endothelial Cells***

On day 1, human umbilical vein endothelial cells (HUVEC) were seeded at  $2.5 \times 10^4$  cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnology, Inc.). On day 2, the medium was replaced with M199 containing 10% FBS, 8 units/ml heparin. VEGF2 protein of SEQ ID NO. 2 minus the initial 45 amino acid residues, (VEGF) and basic FGF (bFGF) were added, at the concentration shown. On days 4 and 6, the medium was replaced. On day 8, cell number was determined with a Coulter Counter (See Figure 12).

***Example 6***

***The Effect of Purified VEGF2 Protein on the Growth of Vascular Endothelial Cells***

On day 1, human umbilical vein endothelial cells (HUVEC) were seeded at  $2.5 \times 10^4$  cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, 50 units/ml endothelial cell growth supplements (ECGS, Biotechnology, Inc.). On day 2, the medium was replaced with M199 containing 10% FBS, 8 units/ml heparin. Purified VEGF2 protein of SEQ ID NO:2 minus initial 45 amino acid residues was added to the medium at this point. On days 4 and 6, the medium was replaced with fresh medium and supplements. On day 8, cell number was determined with a Coulter Counter (See Figure 13).

***Example 7***

### *Expression via Gene Therapy*

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. *et al.*, *DNA* 7:219-225 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer further includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the



vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

5 Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as *neo* or *his*.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

### Example 8

#### Expression of VEGF2 mRNA in Human Fetal and Adult Tissues

##### Experimental Design

20 Northern blot analysis was carried out to examine the levels of expression of VEGF2 mRNA in human fetal and adult tissues. A cDNA probe containing the entire nucleotide sequence of the VEGF-2 protein was labeled with <sup>32</sup>P using the rediprime\* DNA labeling system (Amersham Life Science), according to the manufacturer's instructions. After labeling, the probe was purified using a CHROMA SPIN-100\* column (Clontech Laboratories, Inc.), according to  
25 manufacturer's protocol number PT1200-1. The purified labeled probe was then used to examine various human tissues for VEGF-2 mRNA.

A Multiple Tissue Northern (MTN) blot containing various human tissues (Fetal Kidney, Fetal Lung, Fetal Liver, Brain, Kidney, Lung, Liver, Spleen, Thymus, Bone Marrow, Testes, Placenta, and Skeletal Muscle) was obtained from Clontech. The MTN blot was examined with the labeled probe using ExpressHyb\* hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blot was exposed to film at -70°C overnight with an intensifying screen and developed according to standard procedures.

## Results

Expression of VEGF2 mRNA is abundant in vascular smooth muscle and several highly vascularized tissues. VEGF2 is expressed at significantly higher levels in tissues associated with hematopoietic or angiogenic activities, i.e. fetal kidney, fetal lung, bone marrow, placental, spleen and lung tissue. The expression level of VEGF2 is low in adult kidney, fetal liver, adult liver, testes; and is almost undetectable in fetal brain, and adult brain (See FIG. 14).

In primary cultured cells, the expression of VEGF-2 mRNA is abundant in vascular smooth muscle cells and dermal fibroblast cells, but much lower in human umbilical vein endothelial cells (see FIG. 15). This mRNA distribution pattern is very similar to that of VEGF.

### *Example 9*

#### *Construction of Amino terminal and carboxy terminal deletion mutants*

In order to identify and analyze biologically active VEGF-2 polypeptides, a panel of deletion mutants of VEGF-2 was constructed using the expression vector pHE4a.

#### 1. Construction of VEGF-2 T103-L215 in pHE4

To permit Polymerase Chain Reaction directed amplification and sub-cloning of VEGF-2 T103-L215 (amino acids 103 to 215 in Figure 1 or SEQ ID NO:18) into the E.coli protein expression vector, pHE4, two oligonucleotide primers complementary to the desired region of VEGF2 were synthesized with the following base sequence:

5' Primer (Nde I/START and 18 nt of coding sequence):

5'-GCA GCA CAT ATG ACA GAA GAG ACT ATA AAA-3' (SEQ ID NO: 19)

3' Primer (Asp718, STOP, and 15 nt of coding sequence):

5'-GCA GCA GGT ACC TCA CAG TTT AGA CAT GCA-3' (SEQ ID NO: 20)

The above described 5' primer (SEQ ID NO: 19), incorporates an NdeI restriction site and the above described 3' Primer (SEQ ID NO:20), incorporates an Asp718 restriction site. The 5' primer (SEQ ID NO:19) also contains an ATG sequence adjacent and in frame with the VEGF-2 coding region to allow translation of the cloned fragment in E.coli, while the 3' primer (SEQ ID NO:20) contains one stop codon (preferentially utilized in E.coli) adjacent and in frame with the VEGF-2 coding region which ensures correct translational termination in E.coli.

The Polymerase Chain Reaction was performed using standard conditions well known to those skilled in the art and the nucleotide sequence for the mature VEGF-2 (aa 24-419 in SEQ ID NO:18) as, for example, constructed in Example 3 as template. The resulting amplicon was restriction digested with NdeI and Asp718 and subcloned into NdeI/Asp718 digested pHE4a expression vector.

## 2. C nstructi n f VEGF-2 T103-R227 in pHE4

To permit Polymerase Chain Reaction directed amplification and sub-cloning of VEGF-2 T103-R227 (amino acids 103 to 227 in Figure 1 or SEQ ID NO:18) into the E.coli protein expression vector, pHE4, two oligonucleotide primers complementary to the desired region of VEGF2 were synthesized with the following base sequence:

5' Primer (Nde I/START and 18 nt of coding sequence):

5'-GCA GCA CAT ATG ACA GAA GAG ACT ATA AAA-3' (SEQ ID NO:19)

3' Primer (Asp 718, STOP, and 15 nt of coding sequence):

5'-GCA GCA GGT ACC TCA ACG TCT AAT AAT GGA-3' (SEQ ID NO:21)

In the case of the above described primers, an NdeI or Asp718 restriction site was incorporated into the 5' primer and 3' primer, respectively. The 5' primer (SEQ ID NO:19) also contains an ATG sequence adjacent and in frame with the VEGF-2 coding region to allow translation of the cloned fragment in E.coli, while the 3' Primer (SEQ ID NO:21) contains one stop codon (preferentially utilized in E.coli) adjacent and in frame with the VEGF-2 coding region which ensures correct translational termination in E.coli.

The Polymerase Chain Reaction was performed using standard conditions well known to those skilled in the art and the nucleotide sequence for the mature VEGF-2 (aa 24-419 in SEQ ID NO:18) as, for example, constructed in Example 3, as template. The resulting amplicon was restriction digested with NdeI and Asp718 and subcloned into NdeI/Asp718 digested pHE4a protein expression vector.

### 3. Construction of VEGF-2 T103-L215 in pA2GP

In this illustrative example, the plasmid shuttle vector pA2 GP is used to insert the cloned DNA encoding the N-terminal and C-terminal deleted VEGF-2 protein (amino acids 103-215 in Figure 1 or SEQ ID NO:18), into a baculovirus to express the N-terminal and C-terminal deleted VEGF-2 protein, using a baculovirus leader and standard methods as described in Summers *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by the secretory signal peptide (leader) of the baculovirus gp67 protein and convenient restriction sites such as BamHI, Xba I and Asp718. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak *Drosophila* promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that expresses the cloned polynucleotide.

Many other baculovirus vectors could be used in place of the vector above, such as pAc373, pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required. Such vectors are described, for instance, in Luckow *et al.*, *Virology* 170:31-39 (1989).

The cDNA sequence encoding the VEGF-2 protein without 102 amino acids at the N-terminus and without 204 amino acids at the C-terminus in Figure 1, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene.

The 5' primer has the sequence 5'-GCA GCA GGA TCC CAC AGA AGA GAC TAT AAA- 3' (SEQ ID NO:22) containing the BamHI restriction enzyme site

(in bold) followed by 1 spacer nt to stay in-frame with the vector-supplied signal peptide, and 17 nt of coding sequence bases of VEGF-2 protein. The 3' primer has the sequence 5'-GCA GCA TCT AGA TCA CAG TTT AGA CAT GCA-3' (SEQ ID NO:23) containing the XbaI restriction site (in bold) followed by a stop codon and 17 nucleotides complementary to the 3' coding sequence of VEGF-2.

The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("GeneClean," BIO 101, Inc., La Jolla, CA). The fragment was then digested with the endonuclease BamHI and XbaI and then purified again on a 1% agarose gel. This fragment was ligated to pA2 GP baculovirus transfer vector (Supplier) at the BamHI and XbaI sites. Through this ligation, VEGF2 cDNA representing the N-terminal and C-terminal deleted VEGF-2 protein (amino acids 103-215 in Figure 1 or SEQ ID NO:18) was cloned in frame with the signal sequence of baculovirus GP gene and was located at the 3' end of the signal sequence in the vector. This is designated pA2GPVEGF2.T103-L215.

#### 4. Construction of VEGF-2 T103-R227 in pA2GP

The cDNA sequence encoding the VEGF-2 protein without 102 amino acids at the N-terminus and without 192 amino acids at the C-terminus in Figure 1 (i.e., amino acids 103-227 of SEQ ID NO:18) was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene.

The 5'-GCA GCA GGA TCC CAC AGA AGA GAC TAT AAA ATT TGC TGC-3' primer has the sequence (SEQ ID NO:24) containing the BamHI restriction enzyme site (in bold) followed by 1 spacer nt to stay in-frame with the vector-supplied signal peptide, and 26 nt of coding sequence bases of VEGF-2 protein. The 3' primer has the sequence 5'-GCA GCA TCT AGA TCA ACG TCT AAT AAT GGA ATG AAC-3' (SEQ ID NO:25) containing the XbaI restriction site (in bold) followed by a stop codon and 21 nucleotides complementary to the 3' coding sequence of VEGF-2.

The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101, Inc., La Jolla, CA). The fragment was then digested with the endonuclease BamHI and XbaI and then purified again on a 1% agarose gel. This fragment was ligated to pA2 GP baculovirus transfer vector (Supplier) at the BamHI and XbaI sites. Through this ligation, VEGF2 cDNA representing the N-terminal and C-terminal deleted VEGF-2 protein (amino acids 103-227 in Figure 1 or SEQ ID NO:18) was cloned in frame with the signal sequence of baculovirus GP gene and was located at the 3' end of the signal sequence in the vector. This construct is designated pA2GPVEGF2.T103-R227.

### 5. Construction of VEGF-2 in pC1

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al.*, *Molecular and Cellular Biology*, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart *et al.*, *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

The vector pC1 is used for the expression of VEGF-2 protein. Plasmid pC1 is a derivative of the plasmid pSV2-dhfr [ATCC Accession No. 37146]. Both plasmids contain the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F.W., Kellems, R.M., Bertino, J.R., and Schimke, R.T., 1978, *J. Biol. Chem.* 253:1357-1370, Hamlin, J.L. and Ma, C. 1990, *Biochem. et Biophys. Acta*,

1097:107-143, Page, M.J. and Sydenham, M.A. 1991, Biotechnology Vol. 9:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene it is usually co-amplified and over-expressed. It is state of the art to develop cell lines carrying more than 1,000 copies of the genes. Subsequently, when the methotrexate is withdrawn, cell lines contain the amplified gene integrated into the chromosome(s).

Plasmid pC1 contains for the expression of the gene of interest a strong promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus (Cullen, *et al.*, Molecular and Cellular Biology, March 1985:438-4470) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart *et al.*, Cell 41:521-530, 1985). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: BamHI, PvuII, and NruI. Behind these cloning sites the plasmid contains translational stop codons in all three reading frames followed by the 3' intron and the polyadenylation site of the rat preproinsulin gene. Other high efficient promoters can also be used for the expression, e.g., the human  $\beta$ -actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLV. For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well.

Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC1 is digested with the restriction enzyme BamHI and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding VEGF-2, ATCC Accession No. 97149, was constructed by PCR using two primers corresponding to the 5' and 3' ends of the VEGF-2 gene: the 5' Primer (5'-GAT CGA TCC ATC ATG CAC TCG CTG



GGC TTC TTC TCT GTG GCG TGT TCT CTG CTC G-3' (SEQ ID NO:26)) contains a Klenow-filled BamHI site and 40 nt of VEGF-2 coding sequence starting from the initiation codon; the 3' primer (5'-GCA GGG TAC GGA TCC TAG ATT AGC TCA TTT GTG GTC TTT-3' (SEQ ID NO:27)) contains a BamHI site and 16 nt of VEGF-2 coding sequence not including the stop codon.

The PCR amplified DNA fragment is isolated from a 1% agarose gel as described above and then digested with the endonuclease BamHI and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 cells are then transformed and bacteria identified that contained the plasmid pC1. The sequence and orientation of the inserted gene is confirmed by DNA sequencing. This construct is designated pC1VEGF-2.

## 6. Construction of pC4SigVEGF-2 T103-L215

### Insert description for pC4Sig

Plasmid pC4Sig is plasmid pC4 (Accession No. 209646) containing a human IgG Fc portion as well as a protein signal sequence.

To permit Polymerase Chain Reaction directed amplification and sub-cloning of VEGF-2 T103-L215 (amino acids 103 to 215 in Figure 1 or SEQ ID NO:18) into pC4Sig, two oligonucleotide primers complementary to the desired region of VEGF2 were synthesized with the following base sequence:

5' Primer (Bam HI and 26 nt of coding sequence):

5'-GCA GCA GGA TCC ACA GAA GAG ACT ATA AAA TTT GCT GC-3'  
SEQ ID NO: 34

3' Primer (Xba I, STOP, and 15 nt of coding sequence):

5'-CGT CGT TCT AGA TCA CAG TTT AGA CAT GCA TCG GCA G-3' SEQ  
ID NO:35

The Polymerase Chain Reaction was performed using standard conditions well known to those skilled in the art and the nucleotide sequence for the mature VEGF-2 (aa 24-419) as, for example, constructed in Example 3, as template. The resulting amplicon was restriction digested with BamHI and XbaI and subcloned into BamHI /XbaI digested pC4Sig vector.

#### 7. Construction of pC4SigVEGF-2 T103-R227

To permit Polymerase Chain Reaction directed amplification and sub-cloning of VEGF-2 T103-L215 (amino acids 103 to 227 in Figure 1 or SEQ ID NO:18) into pC4Sig, two oligonucleotide primers complementary to the desired region of VEGF2 were synthesized with the following base sequence:

5' Primer (Bam HI and 26 nt of coding sequence):

5'-GCA GCA GGA TCC ACA GAA GAG ACT ATA AAA TTT GCT GC-3' SEQ ID NO: 34

3' Primer (Xba I, STOP, and 21 nt of coding sequence):

5'-GCA GCA TCT AGA TCA ACG TCT AAT AAT GGA ATG AAC-3' SEQ ID NO:25

The Polymerase Chain Reaction was performed using standard conditions well known to those skilled in the art and the nucleotide sequence for the mature VEGF-2 (aa 24-419) as, for example, constructed in Example 3, as template. The resulting amplicon was restriction digested with BamHI and XbaI and subcloned into BamHI /XbaI digested pC4Sig vector.

#### 8. Construction of pC4VEGF-2 M1-M263

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T01280-9275260

The expression vector pC4 contains the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al.*, *Molecular and Cellular Biology*, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart *et al.*, *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vector contains in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

In this illustrative example, the cloned DNA encoding the C-terminal deleted VEGF-2 M1-M263 protein (amino acids 1-263 in Figure 1 or SEQ ID NO:18) is inserted into the plasmid vector pC4 to express the C-terminal deleted VEGF-2 protein.

To permit Polymerase Chain Reaction directed amplification and sub-cloning of VEGF-2 M1-M263 into the expression vector, pC4, two oligonucleotide primers complementary to the desired region of VEGF2 were synthesized with the following base sequence:

5' Primer 5'-GAC TGG ATC CGC CAC CAT GCA CTC GCT GGG CTT CTT CTC-3' (SEQ ID NO:28)

3' Primer 5'-GAC TGG TAC CTT ATC ACA TAA AAT CTT CCT GAG CC-3' (SEQ ID NO: 29)

In the case of the above described 5' primer, an BamHI restriction site was incorporated, while in the case of the 3' primer, an Asp718 restriction site was incorporated. The 5' primer also contains 6 nt, 20 nt of VEGF-2 coding sequence, and an ATG sequence adjacent and in frame with the VEGF-2 coding region to allow translation of the cloned fragment in E.coli, while the 3' primer contains 2 nt, 20 nt of VEGF-2 coding sequence, and one stop codon (preferentially utilized in E.coli) adjacent and in frame with the VEGF-2 coding region which ensures correct translational termination in E.coli.

The Polymerase Chain Reaction was performed using standard conditions well known to those skilled in the art and the nucleotide sequence for the mature VEGF-2 (aa 24-419) as constructed, for example, in Example 3 as template. The resulting amplicon was restriction digested with BamH1 and Asp718 and subcloned into BamH1/Asp718 digested pC4 protein expression vector. This construct is designated pC4VEGF-2 M1-M263.

### 9. Construction of pC4VEGF-2 M1-D311

In this illustrative example, the cloned DNA encoding the C-terminal deleted VEGF-2 M1-D311 protein (amino acids 1-311 in Figure 1 or SEQ ID NO:18) is inserted into the plasmid vector pC4 to express the C-terminal deleted VEGF-2 protein.

To permit Polymerase Chain Reaction directed amplification and sub-cloning of VEGF-2 M1-D311 into the expression vector, pC4, two oligonucleotide primers complementary to the desired region of VEGF2 were synthesized with the following base sequence:

5' Primer 5'-GAC TGG ATC CGC CAC CAT GCA CTC GCT GGG CTT CTT CTC-3' (SEQ ID NO:30)

3' Primer 5'-GAC TGG TAC CTT ATC AGT CTA GTT CTT TGT GGG G-3' (SEQ ID NO: 31)

In the case of the above described 5' primer, an BamH1 restriction site was incorporated, while in the case of the 3' primer, an Asp718 restriction site was incorporated. The 5' primer also contains 6 nt, 20 nt of VEGF-2 coding sequence, and an ATG sequence adjacent and in frame with the VEGF-2 coding region to allow translation of the cloned fragment in E.coli, while the 3' primer contains 2 nt, 20 nt of VEGF-2 coding sequence, and one stop codon (preferentially utilized

in E.coli) adjacent and in frame with the VEGF-2 coding region which ensures correct translational termination in E.coli.

The Polymerase Chain Reaction was performed using standard conditions well known to those skilled in the art and the nucleotide sequence for the mature VEGF-2 (aa 24-419) as constructed, for example, in Example 3 as template. The resulting amplicon was restriction digested with BamH1 and Asp718 and subcloned into BamH1/Asp718 digested pC4 protein expression vector.

#### 10. Construction of pC4VEGF-2 M1-Q367

In this illustrative example, the cloned DNA encoding the C-terminal deleted VEGF-2 M1-D311 protein (amino acids 1-311 in SEQ ID NO:18) is inserted into the plasmid vector pC4 to express the C-terminal deleted VEGF-2 protein.

To permit Polymerase Chain Reaction directed amplification and sub-cloning of VEGF-2 M1-D311 into the expression vector, pC4, two oligonucleotide primers complementary to the desired region of VEGF2 were synthesized with the following base sequence:

5' Primer 5'-GAC TGG ATC CGC CAC CAT GCA CTC GCT GGG CTT CTT CTC-3' (SEQ ID NO:32)

3' Primer 5'-GAC TGG TAC CTC ATT ACT GTG GAC TTT CTG TAC ATT C-3' (SEQ ID NO:33)

In the case of the above described 5' primer, an BamH1 restriction site was incorporated, while in the case of the 3' primer, an Asp718 restriction site was incorporated. The 5' primer also contains 6 nt, 20 nt of VEGF-2 coding sequence, and an ATG sequence adjacent and in frame with the VEGF-2 coding region to

allow translation of the cloned fragment in E.coli, while the 3' primer contains 2 nt, 20 nt of VEGF-2 coding sequence, and one stop codon (preferentially utilized in E.coli) adjacent and in frame with the VEGF-2 coding region which ensures correct translational termination in E.coli.

5 The Polymerase Chain Reaction was performed using standard conditions well known to those skilled in the art and the nucleotide sequence for the mature VEGF-2 (aa 24-419) as constructed, for example, in Example 3 as template. The resulting amplicon was restriction digested with BamH1 and Asp718 and subcloned into BamH1/Asp718 digested pC4 protein expression vector. This construct is designated pC4VEGF-2 M1-Q367.

### Example 10

#### *Transient Expression of VEGF-2 Protein in COS-7 Cells*

##### **Experimental Design**

15  
20  
25  
Expression of the VEGF-2-HA fusion protein from the construct made in Example 4, for example, was detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow and colleagues (Antibodies: A Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988)). To this end, two days after transfection, the cells were labeled by incubation in media containing 35S-cysteine for 8 hours. The cells and the media were collected, and the cells were washed and then lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson and colleagues (supra). Proteins were precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then were analyzed by SDS-PAGE and autoradiography.

## Results

As shown in FIG. 16, cells transfected with pcDNA1 VEGF-2HA secreted a 56 kd and a 30 kd protein. The 56 kd protein, but not the 30 kd protein, could also be detected in the cell lysate but is not detected in controls. This suggests the 30 kd protein is likely to result from cleavage of the 56 kd protein. Since the HA-tag is on the C-terminus of VEGF-2, the 30 kd protein must represent the C-terminal portion of the cleaved protein, whereas the N-terminal portion of the cleaved protein would not be detected by immunoprecipitation. These data indicate that VEGF-2 protein expressed in mammalian cells is secreted and processed.

### *Example 11*

#### *Stimulatory effect of VEGF2 on proliferation of vascular endothelial cells*

##### **Experimental Design**

Expression of VEGF2 is abundant in highly vascularized tissues. Therefore the role of VEGF2 in regulating proliferation of several types of endothelial cells was examined.

##### ***Endothelial cell proliferation assay***

For evaluation of mitogenic activity of growth factors, the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium) assay with the electron coupling reagent PMS (phenazine methosulfate) was performed (CellTiter 96 AQ, Promega). Cells were seeded in a 96-well plate (5,000 cells/well) in 0.1 mL serum-supplemented medium and allowed to attach overnight. After serum-starvation for 12 hours in 0.5% FBS, conditions (bFGF, VEGF<sub>165</sub> or VEGF-2 in 0.5% FBS) with or without Heparin (8 U/ml) were added to wells for 48 hours. 20 µg of MTS/PMS mixture (1:0.05) were added per well and allowed to incubate for 1 hour at 37°C before measuring

the absorbance at 490 nm in an ELISA plate reader. Background absorbance from control wells (some media, no cells) was subtracted, and seven wells were performed in parallel for each condition. See, Leak *et al. In Vitro Cell. Dev. Biol.* 30A:512-518 (1994)

## Results

VEGF2 stimulated proliferation of human umbilical vein endothelial cells (HUVEC) and dermal microvascular endothelial cells slightly (FIGs. 17 and 18). The stimulatory effect of VEGF2 is more pronounced on proliferation of endometrial and microvascular endothelial cells (FIG. 19). Endometrial endothelial cells (HEEC) demonstrated the greatest response to VEGF2 (96% of the effect of VEGF on microvascular endothelial cells). The response of microvascular endothelial cells (HMEC) to VEGF2 was 73% compared to VEGF. The response of HUVEC and BAEC (bovine aortic endothelial cells) to VEGF2 was substantially lower at 10% and 7%, respectively. The activity of VEGF2 protein has varied between different purification runs with the stimulatory effect of certain batches on HUVEC proliferation being significantly higher than that of other batches.

### Example 12

#### *Inhibition of PDGF-induced vascular smooth muscle cell proliferation*

VEGF2 expression is high in vascular smooth muscle cells. Smooth muscle is an important therapeutic target for vascular diseases, such as restenosis. To evaluate the potential effects of VEGF2 on smooth muscle cells, the effect of VEGF2 on human aortic smooth muscle cell (HAoSMC) proliferation was examined.

#### Experimental Design

HAoSMC proliferation can be measured, for example, by BrdUrd incorporation. Briefly, subconfluent, quiescent cells grown on the 4-chamber slides



are transfected with CRP or FITC-labeled AT2-3LP. Then, the cells are pulsed with 10% calf serum and 6  $\mu\text{g/ml}$  BrdUrd. After 24 h, immunocytochemistry is performed by using BrdUrd Staining Kit (Zymed Laboratories). In brief, the cells are incubated with the biotinylated mouse anti-BrdUrd antibody at 4 °C for 2 h after exposing to denaturing solution and then with the streptavidin-peroxidase and diaminobenzidine. After counterstaining with hematoxylin, the cells are mounted for microscopic examination, and the BrdUrd-positive cells are counted. The BrdUrd index is calculated as a percent of the BrdUrd-positive cells to the total cell number. In addition, the simultaneous detection of the BrdUrd staining (nucleus) and the FITC uptake (cytoplasm) is performed for individual cells by the concomitant use of bright field illumination and dark field-UV fluorescent illumination. See, Hayashida *et al.*, *J. Biol. Chem.* 6;271(36):21985-21992 (1996).

## Results

VEGF2 has an inhibitory effect on proliferation of vascular smooth muscle cells induced by PDGF, but not by Fetal Bovine Serum (FBS) (FIG. 20).

## Example 13

### *Stimulation of endothelial cell migration*

Endothelial cell migration is an important step involved in angiogenesis.

## Experimental Design

This example will be used to explore the possibility that VEGF-2 may stimulate lymphatic endothelial cell migration. Currently, there are no published reports of such a model. However, we will be adapting a model of vascular endothelial cell migration for use with lymphatic endothelial cells

essentially as follows:

Endothelial cell migration assays are performed using a 48 well microchemotaxis chamber (Neuroprobe Inc., Cabin John, MD; Falk, W., Goodwin, R. H. J., and Leonard, E. J. "A 48 well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration." J. Immunological Methods 1980;33:239-247). Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8  $\mu$ m (Nucleopore Corp. Cambridge, MA) are coated with 0.1% gelatin for at least 6 hours at room temperature and dried under sterile air. Test substances are diluted to appropriate concentrations in M199 supplemented with 0.25% bovine serum albumin (BSA), and 25  $\mu$ l of the final dilution is placed in the lower chamber of the modified Boyden apparatus. Subconfluent, early passage (2-6) HUVEC or BMEC cultures are washed and trypsinized for the minimum time required to achieve cell detachment. After placing the filter between lower and upper chamber,  $2.5 \times 10^5$  cells suspended in 50  $\mu$ l M199 containing 1% FBS are seeded in the upper compartment. The apparatus is then incubated for 5 hours at 37°C in a humidified chamber with 5% CO<sub>2</sub> to allow cell migration. After the incubation period, the filter is removed and the upper side of the filter with the non-migrated cells is scraped with a rubber policeman. The filters are fixed with methanol and stained with a Giemsa solution (Diff-Quick, Baxter, McGraw Park, IL). Migration is quantified by counting cells of three random high-power fields (40x) in each well, and all groups are performed in quadruplicate.

## Results

In an assay examining HUVEC migration using a 48-well microchemotaxis chamber, VEGF2 was able to stimulate migration of HUVEC (Fig. 21).

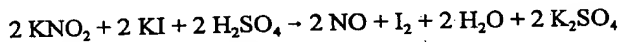
**Example 14*****Stimulation of nitric oxide production by endothelial cells***

Nitric oxide released by the vascular endothelium is believed to be a mediator of vascular endothelium relaxation. VEGF-1 has been demonstrated to induce nitric oxide production by endothelial cells in response to VEGF-1. As a result, VEGF-2 activity can be assayed by determining nitric oxide production by endothelial cells in response to VEGF-2.

**Experimental Design**

Nitric oxide is measured in 96-well plates of confluent microvascular endothelial cells after 24 hours starvation and a subsequent 4 hr exposure to various levels of VEGF-1 and VEGF-2. Nitric oxide in the medium is determined by use of the Griess reagent to measure total nitrite after reduction of nitric oxide-derived nitrate by nitrate reductase. The effect of VEGF2 on nitric oxide release was examined on HUVEC.

Briefly, NO release from cultured HUVEC monolayer was measured with a NO-specific polarographic electrode connected to a NO meter (Iso-NO, World Precision Instruments Inc.) (1049). Calibration of the NO elements was performed according to the following equation:



The standard calibration curve was obtained by adding graded concentrations of  $\text{KNO}_3$  (0, 5, 10, 25, 50, 100, 250, and 500 nmol/L) into the calibration solution containing KI and  $\text{H}_2\text{SO}_4$ . The specificity of the Iso-NO electrode to NO was previously determined by measurement of NO from authentic NO gas (1050). The culture medium was removed and HUVECs were washed twice with Dulbecco's phosphate buffered saline. The cells were then bathed in 5 ml of filtered Krebs-Henseleit solution in 6-well plates, and the cell plates were kept on a slide warmer (Lab Line Instruments Inc.) To maintain the temperature at 37 °C. The NO sensor probe was inserted vertically into the wells, keeping the tip of the electrode 2 mm

under the surface of the solution, before addition of the different conditions. S-nitroso acetyl penicillamin (SNAP) was used as a positive control. The amount of released NO was expressed as picomoles per  $1 \times 10^6$  endothelial cells. All values reported were means of four to six measurements in each group (number of cell culture wells). See, Leak *et al. Biochem. and Biophys. Res. Comm.* 217:96-105 (1995).

## Results

VEGF-2 was capable of stimulating nitric oxide release on HUVEC (FIG. 22) to a higher level than VEGF. This suggested that VEGF2 may modify vascular permeability and vessel dilation.

### Example 15

#### *Effect of VEGF-2 on cord formation in angiogenesis*

Another step in angiogenesis is cord formation, marked by differentiation of endothelial cells. This bioassay measures the ability of microvascular endothelial cells to form capillary-like structures (hollow structures) when cultured *in vitro*.

#### Experimental Design

CADMEC (microvascular endothelial cells) are purchased from Cell Applications, Inc. as proliferating (passage 2) cells and are cultured in Cell Applications' CADMEC Growth Medium and used at passage 5. For the *in vitro* angiogenesis assay, the wells of a 48-well cell culture plate are coated with Cell Applications' Attachment Factor Medium (200  $\mu$ l/well) for 30 min. at 37°C. CADMEC are seeded onto the coated wells at 7,500 cells/well and cultured overnight in Growth Medium. The Growth Medium is then replaced with 300  $\mu$ g Cell Applications' Cord Formation Medium containing control buffer or HGS protein (0.1 to 100 ng/ml) and the cells are cultured for an additional 48 hr. The

numbers and lengths of the capillary-like chords are quantitated through use of the Boeckeler VIA-170 video image analyzer. All assays are done in triplicate.

Commercial (R&D) VEGF (50 ng/ml) is used as a positive control.  $\beta$ -esteradiol (1 ng/ml) is used as a negative control. The appropriate buffer (without protein) is also utilized as a control.

## Results

It has been observed that VEGF2 inhibits cord formation similar to IFN $\alpha$  which also stimulates endothelial cell proliferation (FIG. 23). This inhibitory effect may be a secondary effect of endothelial proliferation which is mutually exclusive with the cord formation process.

### *Example 16*

#### *Angiogenic effect on chick chorioallantoic membrane*

Chick chorioallantoic membrane (CAM) is a well-established system to examine angiogenesis. Blood vessel formation on CAM is easily visible and quantifiable. The ability of VEGF2 to stimulate angiogenesis in CAM was examined.

## Experimental Design

### *Embryos*

Fertilized eggs of the White Leghorn chick (*Gallus gallus*) and the Japanese quail (*Coturnix coturnix*) were incubated at 37.8°C and 80% humidity. Differentiated CAM of 16-day-old chick and 13-day-old quail embryos was studied with the following methods.

### CAM Assay

On Day 4 of development, a window was made into the egg shell of chick eggs. The embryos were checked for normal development and the eggs sealed with cellotape. They were further incubated until Day 13. Thermanox coverslips (Nunc, Naperville, IL) were cut into disks of about 5 mm in diameter. Sterile and salt-free growth factors were dissolved in distilled water and about 3.3  $\mu\text{g}/5 \mu\text{l}$  was pipetted on the disks. After air-drying, the inverted disks were applied on CAM. After 3 days, the specimens were fixed in 3% glutaraldehyde and 2% formaldehyde and rinsed in 0.12 M sodium cacodylate buffer. They were photographed with a stereo microscope [Wild M8] and embedded for semi- and ultrathin sectioning as described above. Controls were performed with carrier disks alone.

### Results

This data demonstrates that VEGF2 can stimulate angiogenesis in the CAM assay nine-fold compared to the untreated control. However, this stimulation is only 45% of the level of VEGF stimulation (FIG. 24).

### Example 17

#### *Angiogenesis assay using a Matrigel implant in mouse*

### Experimental Design

In order to establish an *in vivo* model for angiogenesis to test protein activities, mice and rats have been implanted subcutaneously with methylcellulose disks containing either 20 mg of BSA (negative control) and 1 mg of bFGF and 0.5 mg of VEGF-1 (positive control).

It appeared as though the BSA disks contained little vascularization, while the positive control disks showed signs of vessel formation. At day 9, one mouse showed a clear response to the bFGF.

## Results

Both VEGF proteins appeared to enhance Matrigel cellularity by a factor of approximately 2 by visual estimation.

An additional 30 mice were implanted with disks containing BSA, bFGF, and varying amounts of VEGF-1, VEGF-2-B8, and VEGF-2-C4. Each mouse received two identical disks, rather than one control and one experimental disk.

Samples of all the disks recovered were immunostained with Von Willebrand's factor to detect for the presence of endothelial cells in the disks, and flk-1 and flt-4 to distinguish between vascular and lymphatic endothelial cells. However, definitive histochemical analysis of neovascularization and lymphangiogenesis could not be determined.

### *Example 18*

#### *Rescue of Ischemia in Rabbit Lower Limb Model*

##### **Experimental Design**

To study the in vivo effects of VEGF2 on ischemia, a rabbit hindlimb ischemia model was created by surgical removal of one femoral arteries as described previously (Takeshita, S. et al. Am J. Pathol 147:1649-1660 (1995)). The excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Consequently, blood flow to the ischemic limb is dependent upon collateral vessels originating from the internal iliac artery (Takeshita, S. et al. Am J. Pathol 147:1649-1660 (1995)). An interval of 10 days was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. At 10 day post-operatively (day 0), after performing a baseline angiogram, the internal iliac artery of the ischemic limb was transfected with 500 µg naked VEGF2 expression plasmid by arterial gene transfer technology using a hydrogel-coated balloon catheter as described (Riessen, R. et al. Hum Gene Ther. 4:749-758 (1993); Leclerc, G. et al. J. Clin. Invest. 90: 936-944 (1992)). When

VEGF2 was used in the treatment, a single bolus of 500  $\mu$ g VEGF2 protein or control was delivered into the internal iliac artery of the ischemic limb over a period of 1 min. through an infusion catheter. On day 30, various parameters were measured in these rabbits.

## Results

Both VEGF2 protein (FIG. 25, top panels) and naked expression plasmid (FIG. 25, middle panels) were able to restore the following parameters in the ischemic limb. Restoration of blood flow, angiographic score seem to be slightly more by administration of 500  $\mu$ g plasmid compared with by 500  $\mu$ g protein (FIG. 25, bottom panels). The extent of the restoration is comparable with that by VEGF in separate experiments (data not shown). A vessel dilator was not able to achieve the same effect, suggesting that the blood flow restoration is not simply due to a vascular dilation effect.

### a. BP ratio (FIG. 25a)

The blood pressure ratio of systolic pressure of the ischemic limb to that of normal limb.

### b. Blood Flow and Flow Reserve (FIG. 25b)

Resting FL: the blood flow during un-dilated condition

Max FL: the blood flow during fully dilated condition (also an indirect measure of the blood vessel amount)

Flow Reserve is reflected by the ratio of max FL: resting FL.

### c. Angiographic Score (FIG. 25c)

This is measured by the angiogram of collateral vessels. A score was determined by the percentage of circles in an overlaying grid that with crossing opacified arteries divided by the total number in the rabbit thigh.



d. *Capillary density (FIG. 25d)*

The number of collateral capillaries determined in light microscopic sections taken from hindlimbs.

As discussed, VEGF2 is processed to an N-terminal and a C-terminal fragment which are co-purified. The N-terminal fragment contains the intact putative functional domain and may be responsible for the biologic activity.

**Example 19**

*Effect of VEGF2 on Vasodilation*

As described above, VEGF2 can stimulate NO release, a mediator of vascular endothelium dilation. Since dilation of vascular endothelium is important in reducing blood pressure, the ability of VEGF2 to affect the blood pressure in spontaneously hypertensive rats (SHR) was examined. VEGF2 caused a dose-dependent decrease in diastolic blood pressure (FIGs. 26a and b). There was a steady decline in diastolic blood pressure with increasing doses of VEGF2 which attained statistical significance when a dose of 300 µg/kg was administered. The changes observed at this dose were not different than those seen with acetylcholine (0.5 µg/kg). Decreased mean arterial pressure (MAP) was observed as well (FIG. 26c and d). VEGF2 (300 µg/kg) and acetylcholine reduced the MAP of these SHR animals to normal levels.

Additionally, increasing doses (0, 10, 30, 100, 300, and 900 µg/kg) of the B8, C5, and C4 preps of VEGF-2 were administered to 13-14 week old spontaneously hypertensive rats (SHR). Data are expressed as the mean +/- SEM. Statistical analysis was performed with a paired t-test and statistical significance was defined as  $p < 0.05$  vs. the response to buffer alone.

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104280-19275E660  
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Studies with VEGF-2 (C5 prep) revealed that although it significantly decreased the blood pressure, the magnitude of the response was not as great as that seen with VEGF-2 (B8 prep) even when used at a dose of 900 µg/kg.

Studies with VEGF-2 (C4 preparation) revealed that this CHO expressed protein preparation yielded similar results to that seen with C5 (i.e. statistically significant but of far less magnitude than seen with the B8 preparation) (see Figures 26A-D).

As a control and since the C4 and C5 batches of VEGF-2 yielded minor, but statistically significant, changes in blood pressure, experiments were performed with another CHO-expressed protein, M-CIF. Administration of M-CIF at doses ranging from 10-900 µg/kg produced no significant changes in diastolic blood pressure. A minor statistically significant reduction in mean arterial blood pressure was observed at doses of 100 and 900 µg/kg but no dose response was noted. These results suggest that the reductions in blood pressure observed with the C4 and C5 batches of VEGF-2 were specific, i.e. VEGF-2 related.

## Example 20

### Rat Ischemic Skin Flap Model

#### Experimental Design

The evaluation parameters include skin blood flow, skin temperature, and factor VIII immunohistochemistry or endothelial alkaline phosphatase reaction. VEGF-2 expression, during the skin ischemia, is studied using in situ hybridization.

The study in this model is divided into three parts as follows:

- a) Ischemic skin
- b) Ischemic skin wounds
- c) Normal wounds

The experimental protocol includes:

a) Raising a 3x4 cm, single pedicle full-thickness random skin flap (myocutaneous flap over the lower back of the animal).

b) An excisional wounding (4-6 mm in diameter) in the ischemic skin (skin-flap).

c) Topical treatment with VEGF-2 of the excisional wounds (day 0, 1, 2, 3, 4 post-wounding) at the following various dosage ranges: 1 µg to 100 µg.

d) Harvesting the wound tissues at day 3, 5, 7, 10, 14 and 21 post-wounding for histological, immunohistochemical, and in situ studies.

### Example 22

#### Peripheral Arterial Disease Model

Angiogenic therapy using VEGF-2 has been developed as a novel therapeutic strategy to obtain restoration of blood flow around the ischemia in case of peripheral arterial diseases.

#### Experimental Design

The experimental protocol includes:

- a) One side of the femoral artery is ligated to create ischemic muscle of the hindlimb, the other side of hindlimb serves as a control.
- b) VEGF-2 protein, in a dosage range of 20 µg - 500 µg, is delivered intravenously and/or intramuscularly 3 times (perhaps more) per week for 2-3 weeks.
- c) The ischemic muscle tissue is collected after ligation of the femoral artery at 1, 2, and 3 weeks for the analysis of VEGF-2 expression and histology. Biopsy is also performed on the other side of normal muscle of the contralateral hindlimb.

**Example 23****Ischemic Myocardial Disease Model**

VEGF-2 is evaluated as a potent mitogen capable of stimulating the development of collateral vessels, and restructuring new vessels after coronary artery occlusion. Alteration of VEGF-2 expression is investigated in situ.

**Experimental Design**

The experimental protocol includes:

- a) The heart is exposed through a left-side thoracotomy in the rat. Immediately, the left coronary artery is occluded with a thin suture (6-0) and the thorax is closed.
- b) VEGF-2 protein, in a dosage range of 20  $\mu$ g - 500  $\mu$ g, is delivered intravenously and/or intramuscularly 3 times (perhaps more) per week for 2-4 weeks.
- c) Thirty days after the surgery, the heart is removed and cross-sectioned for morphometric and in situ analyzes.

**Example 24****Rat Corneal Wound Healing Model**

This animal model shows the effect of VEGF-2 on neovascularization.

**Experimental Design**

The experimental protocol includes:

- a) Making a 1-1.5 mm long incision from the center of cornea into the stromal layer.
- b) Inserting a spatula below the lip of the incision facing the outer corner of the eye.
- c) Making a pocket (its base is 1-1.5 mm from the edge of the eye).
- d) Positioning a pellet, containing 50 $\mu$ g - 500 $\mu$ g VEGF-2, within the pocket.

e) VEGF-2 treatment can also be applied topically to the corneal wounds in a dosage range of 20µg - 500µg (daily treatment for five days).

### Example 25

#### Diabetic Mouse and Glucocorticoid-Impaired Wound Healing Models

##### Experimental Design

The experimental protocol includes:

##### A. *Diabetic db+/db+ mouse model.*

To demonstrate that VEGF-2 accelerates the healing process, the genetically diabetic mouse model of wound healing is used. The full thickness wound healing model in the db+/db+ mouse is a well characterized, clinically relevant and reproducible model of impaired wound healing. Healing of the diabetic wound is dependent on formation of granulation tissue and re-epithelialization rather than contraction (Gartner, M.H. *et al.*, *J. Surg. Res.* 52:389 (1992); Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235 (1990)).

The diabetic animals have many of the characteristic features observed in Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to their normal heterozygous (db+/+m) littermates. Mutant diabetic (db+/db+) mice have a single autosomal recessive mutation on chromosome 4 (db+) (Coleman *et al.* *Proc. Natl. Acad. Sci. USA* 77:283-293 (1982)). Animals show polyphagia, polydipsia and polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or normal insulin levels, and suppressed cell-mediated immunity (Mandel *et al.*, *J. Immunol.* 120:1375 (1978); Debray-Sachs, M. *et al.*, *Clin. Exp. Immunol.* 51(1):1-7 (1983); Leiter *et al.*, *Am. J. of Pathol.* 114:46-55 (1985)). Peripheral neuropathy, myocardial complications, and microvascular lesions, basement membrane thickening and glomerular filtration abnormalities have been

described in these animals (Norido, F. *et al.*, *Exp. Neurol.* 83(2):221-232 (1984); Robertson *et al.*, *Diabetes* 29(1):60-67 (1980); Giacomelli *et al.*, *Lab Invest.* 40(4):460-473 (1979); Coleman, D.L., *Diabetes* 31 (Suppl):1-6 (1982)). These homozygous diabetic mice develop hyperglycemia that is resistant to insulin analogous to human type II diabetes (Mandel *et al.*, *J. Immunol.* 120:1375-1377 (1978)).

The characteristics observed in these animals suggests that healing in this model may be similar to the healing observed in human diabetes (Greenhalgh, *et al.*, *Am. J. of Pathol.* 136:1235-1246 (1990)).

### Animals

Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic (db+/+m) heterozygous littermates were used in this study (Jackson Laboratories). The animals were purchased at 6 weeks of age and were 8 weeks old at the beginning of the study. Animals were individually housed and received food and water ad libitum. All manipulations were performed using aseptic techniques. The experiments were conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

### Surgical Wounding

Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., *J. Exp. Med.* 172:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized water. The dorsal region of the animal is shaved and the skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration

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of the experiment. Application of the treatment is given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

VEGF-2 is administered using at a range different doses of VEGF-2, from 4 $\mu$ g to 500 $\mu$ g per wound per day for 8 days in vehicle. Vehicle control groups received 50 $\mu$ L of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology and immunohistochemistry. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

### ***Experimental Design***

Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls) were evaluated: 1) Vehicle placebo control, 2) VEGF-2.

### ***Measurement of Wound Area and Closure***

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total square area of the wound. Contraction is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 was 64mm<sup>2</sup>, the corresponding size of the dermal punch. Calculations were made using the following formula:

[Open area on day 8] - [Open area on day 1] / [Open area on day 1]

### **Histology**

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5µm) and cut using a Reichert-Jung microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds are used to assess whether the healing process and the morphologic appearance of the repaired skin is altered by treatment with KGF-2. This assessment included verification of the presence of cell accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and epidermal maturity (Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235 (1990)). A calibrated lens micrometer is used by a blinded observer.

### **Immunohistochemistry**

#### **Re-epithelialization**

Tissue sections are stained immunohistochemically with a polyclonal rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is used as a positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

#### **Cell Proliferation Marker**

Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer served as a positive tissue control and human brain tissue is used as a negative tissue control. Each specimen included a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8,



the lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation.

### **Statistical Analysis**

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

### **B. Steroid Impaired Rat Model**

The inhibition of wound healing by steroids has been well documented in various *in vitro* and *in vivo* systems (Wahl, S.M. Glucocorticoids and Wound healing. In *Anti-Inflammatory Steroid Action: Basic and Clinical Aspects*. 280-302 (1989); Wahl, S.M. *et al.*, *J. Immunol.* 115: 476-481 (1975); Werb, Z. *et al.*, *J. Exp. Med.* 147:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting angiogenesis, decreasing vascular permeability (Ebert, R.H., *et al.*, *Ann. Intern. Med.* 37:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck, L.S. *et al.*, *Growth Factors.* 5: 295-304 (1991); Haynes, B.F., *et al.*, *J. Clin. Invest.* 61: 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes, B.F., *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, S. M. Glucocorticoids and wound healing. In *Antiinflammatory Steroid Action: Basic and Clinical Aspects*. Academic Press. New York. pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well established phenomenon in rats (Beck, L.S. *et al.*, *Growth Factors.* 5: 295-304 (1991); Haynes, B.F., *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, S. M. Glucocorticoids and wound healing. In *Antiinflammatory Steroid Action: Basic and Clinical Aspects*. Academic Press. New York. pp. 280-302 (1989); Pierce, G.F., *et al.*, *Proc. Natl. Acad. Sci. USA.* 86: 2229-2233 (1989)).

To demonstrate that VEGF-2 can accelerate the healing process, the effects of multiple topical applications of VEGF-2 on full thickness excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

### Animals

Young adult male Sprague Dawley rats weighing 250-300 g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and were 9 weeks old at the beginning of the study. The healing response of rats is impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water *ad libitum*. All manipulations are performed using aseptic techniques. This study is conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

### Surgical Wounding

The wounding protocol is followed according to section A, above. On the day of wounding, animals are anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily measurement on days 1-5 and on day 8 for Figure. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue was no longer visible and the wound is covered by a continuous epithelium.

VEGF-2 is administered using at a range different doses of VEGF-2, from 4µg to 500µg per wound per day for 8 days in vehicle. Vehicle control groups received 50µL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

### *Experimental Design*

Four groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) were evaluated: 1) Untreated group 2) Vehicle placebo control 3) VEGF-2 treated groups.

### *Measurement of Wound Area and Closure*

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total area of the wound. Closure is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 was 64mm<sup>2</sup>, the corresponding size of the dermal punch. Calculations were made using the following formula:

$$[\text{Open area on day 8}] - [\text{Open area on day 1}] / [\text{Open area on day 1}]$$

### *Histology*

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5µm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining was performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin was improved by treatment with VEGF-2. A calibrated lens

micrometer was used by a blinded observer to determine the distance of the wound gap.

### ***Statistical Analysis***

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

### **Example 26**

#### **Specific Peptide Fragments to Generate VEGF-2 Monoclonal Antibodies**

Four specific peptides (designated SP-40, SP-41, SP-42 and SP-43) have been generated. These will be used to generate monoclonal antibodies to analyze VEGF-2 processing. The peptides are shown below:

1. "SP-40": MTVLYPEYWKMY (amino acids 70-81 in SEQ ID NO:18)
2. "SP-41": KSIDNEWKRTQSMPREV (amino acids 120-136( note C->S mutation at position 131) in SEQ ID NO: 18)
3. "SP-42": MSKLDVYRQVHSIIRR (amino acids 212-227 in SEQ ID NO: 18)
4. "SP-43": MFSSDAGDDSTDGFHDI (amino acids 263-279 in SEQ ID NO: 18)

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### Example 27

#### Lymphadema Animal Model

The purpose of this experimental approach is to create an appropriate and consistent lymphedema model for testing the therapeutic effects of VEGF-2 in lymphangiogenesis and re-establishment of the lymphatic circulatory system in the rat hind limb. Effectiveness is measured by swelling volume of the affected limb, quantification of the amount of lymphatic vasculature, total blood plasma protein, and histopathology. Acute lymphedema is observed for 7-10 days. Perhaps more importantly, the chronic progress of the edema is followed for up to 3-4 weeks.

#### Experimental Procedure

Prior to beginning surgery, blood sample was drawn for protein concentration analysis. Male rats weighing approximately ~350g are dosed with Pentobarbital. Subsequently, the right legs were shaved from knee to hip. The shaved area is swabbed with gauze soaked in 70% EtOH. Blood is drawn for serum total protein testing. Circumference and volumetric measurements were made prior to injecting dye into paws after marking 2 measurement levels (0.5 cm above heel, at mid-pt of dorsal paw). The intradermal dorsum of both right and left paws are injected with 0.05 ml of 1% Evan's Blue. Circumference and volumetric measurements are then made following injection of dye into paws.

Using the knee joint as a landmark, a mid-leg inguinal incision is made circumferentially allowing the femoral vessels to be located. Forceps and hemostats are used to dissect and separate the skin flaps. After locating the femoral vessels, the lymphatic vessel that runs along side and underneath the vessel(s) is located. The main lymphatic vessels in this area are then electrically coagulated or suture ligated.

Using a microscope, muscles in back of the leg (near the semitendinosus and adductors) are bluntly dissected. The popliteal lymph node is then located. The 2 proximal and 2 distal lymphatic vessels and distal blood supply of the popliteal node are then and ligated by suturing. The popliteal lymph node, and any accompanying adipose tissue, is then removed by cutting connective tissues. Care was taken to control any mild bleeding resulting from this procedure. After lymphatics were occluded, the skin flaps are sealed by using liquid skin (Vetbond) (AJ Buck). The separated skin edges are sealed to the underlying muscle tissue while leaving a gap of ~0.5 cm around the leg. Skin also may be anchored by suturing to underlying muscle when necessary.

To avoid infection, animals are housed individually with mesh (no bedding). Recovering animals were checked daily through the optimal edematous peak, which typically occurred by day 5-7. The plateau edematous peak was then observed. To evaluate the intensity of the lymphedema, we measured the circumference and volumes of 2 designated places on each paw before operation and daily for 7 days. The effect plasma proteins have on lymphedema and determined if protein analysis is a useful testing perimeter is also investigated. The weights of both control and edematous limbs are evaluated at 2 places. Analysis is performed in a blind manner.

**Circumference Measurements:** Under brief gas anesthetic to prevent limb movement, a cloth tape is used to measure limb circumference. Measurements are done at the ankle bone and dorsal paw by 2 different people then those 2 readings are averaged. Readings are taken from both control and edematous limbs.

**Volumetric Measurements:** On the day of surgery, animals are anesthetized with Pentobarbital and are tested prior to surgery. For daily volumetrics animals are under brief halothane anesthetic (rapid immobilization and quick recovery), both legs are shaved and equally marked using waterproof marker on legs. Legs are first dipped in water, then dipped into instrument to each

marked level then measured by Buxco edema software(Chen/Victor). Data is recorded by one person, while the other is dipping the limb to marked area.

Blood-plasma protein measurements: Blood is drawn, spun, and serum separated prior to surgery and then at conclusion for total protein and  $\text{Ca}^{2+}$  comparison.

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Limb Weight Comparison: After drawing blood, the animal is prepared for tissue collection. The limbs were amputated using a quillitine, then both experimental and control legs were cut at the ligature and weighed. A second weighing is done as the tibio-cacaneal joint was disarticulated and the foot was weighed.

Histological Preparations: The transverse muscle located behind the knee (popliteal) area is dissected and arranged in a metal mold, filled with freezeGel, dipped into cold methylbutane, placed into labeled sample bags at -80C until sectioning. Upon sectioning, the muscle was observed under fluorescent microscopy for lymphatics. Other immuno/histological methods are currently being evaluated.

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Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

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